

**UNIVERSIDADE DE LISBOA**

**FACULDADE DE FARMÁCIA**

**DEPARTAMENTO DE MICROBIOLOGIA E IMUNOLOGIA**



**Molecular and Genomic Epidemiology of Multidrug  
Resistant *Mycobacterium tuberculosis* Isolates in  
Lisbon, Portugal**

**João Ruben Lucas Mota Perdigão**

**DOUTORAMENTO EM FARMÁCIA**

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**João Ruben Lucas Mota Perdigão**

Tese orientada pela Prof. Doutora Isabel Portugal

Tese especialmente elaborada para a obtenção do grau de doutor em Farmácia,  
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*“If it is a terrifying thought that life is at the mercy of the multiplication of these minute bodies, it is a consoling hope that Science will not always remain powerless before such enemies...”*

Louis Pasteur



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## PREFACE

The work that is detailed in this thesis is centered on the theme of multidrug resistant tuberculosis in Portugal. We have aimed at obtaining a better picture of the epidemiology and drug resistance in Lisbon Health Region through the use of molecular biology techniques. This research was mainly conducted in the Unidade dos Retrovírus e Infecções Associadas from the Centro de Patogénese Molecular in Faculdade de Farmácia da Universidade de Lisboa, although a part of it was carried out in the Instituto Nacional de Saúde Dr. Ricardo Jorge.

This research theme is presented along this thesis as a compilation of scientific articles (Chapters 2-7) published in peer-reviewed international journals or yet in a stage of submission/preparation. These scientific articles and its order reflect the evolution in our knowledge concerning strain clonality and the molecular determinants of resistance in Lisbon Health Region.

A General Introduction (Chapter 1) precedes these chapters and is intended to present a global view on some of the tuberculosis topics and its current state of knowledge, which the author found necessary in order to provide the reader with a broader understanding of the research that is discussed in the subsequent chapters. A General Discussion (Chapter 8) follows all chapters as it is intended to summarize the main findings in the previous chapters and to give these some perspective in the light of the evolving knowledge.

This thesis is therefore based in the following manuscripts already published or in preparation:

**Perdigao J**, Macedo R, Joao I, Fernandes E, Brum L, Portugal I. Multidrug-resistant tuberculosis in Lisbon, Portugal: a molecular epidemiological perspective. *Microb Drug Resist* 2008; **14**: 133-43.

**Perdigao J**, Macedo R, Malaquias A, Ferreira A, Brum L, Portugal I. Genetic analysis of extensively drug-resistant *Mycobacterium tuberculosis* strains in Lisbon, Portugal. *J Antimicrob Chemother* 2010; **65**: 224-7.

**Perdigao J**, Macedo R, Silva C, Pinto C, Furtado C, Brum L, Portugal I. Tuberculosis drug-resistance in Lisbon, Portugal: a 6-year overview. *Clin Microbiol Infect* 2011; **17**: 1397-402.

**Perdigao J**, Macedo R, Silva C, Machado D, Couto I, Viveiros M, Jordao L, Portugal I. From multidrug-resistant to extensively drug-resistant tuberculosis in Lisbon, Portugal: the stepwise mode of resistance acquisition. *J Antimicrob Chemother* 2013; **68**: 27-33.

**Perdigao J**, Macedo R, Machado D, Silva C, Jordao L, Couto I, Viveiros M, Portugal I. GidB mutation as a phylogenetic marker for Q1 cluster *Mycobacterium tuberculosis* isolates and intermediate-level streptomycin resistance determinant in Lisbon, Portugal. *Manuscript in preparation*

**Perdigao J**, Silva H, Machado D, Macedo, Maltez F, Silva C, Jordao L, Couto I, Mallard K, Coll F, Hill-Cawthorne G, McNerney R, Pain A, Clark TG, Viveiros M, Portugal I. Unraveling *Mycobacterium tuberculosis* genomic diversity and evolution in Lisbon, Portugal, a highly drug resistant setting. *Manuscript in preparation*

#### **Other publications:**

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**Perdigao J**, Macedo R, Ribeiro A, Brum L, Portugal I. Genetic characterization of the ethambutol resistance-determining region in *Mycobacterium tuberculosis*: prevalence and significance of embB306 mutations. *Int J Antimicrob Agents* 2009; **33**: 334-8.

**Perdigao J**, Milho C, Carrilho L, Brum L, Portugal I. Genotypic analysis of *Mycobacterium tuberculosis* isolates from a Lisbon hospital in Portugal. *Rev Port Pneumol* 2009; **15**: 761-9.

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Oral Communications in International Conferences:

**Perdigão J**, Malaquias C, Macedo R, Brum L, Portugal I. 2008. *Mutational characterization of tlyA gene in capreomycin-resistant Mycobacterium tuberculosis clinical isolates*. 29<sup>th</sup> Annual Congress of the European Society of Mycobacteriology. Plovdiv, Bulgaria

**Perdigão J**, Macedo R, Silva C, Pinto C, Furtado C, Brum L, Portugal I. 2011. *Six-year laboratory data report on drug-resistant tuberculosis in Lisbon Health Region, Portugal*. 21<sup>st</sup> ECCMID/ 27<sup>th</sup> ICC 2011. Milan, Italy.

Oral Communications in National Conferences:

**Perdigão J**, Sabino A, Milho C, Macedo R, Brum L, Portugal I. 2008. *Detecção de mutações no gene gidB em Mycobacterium tuberculosis resistentes à estreptomicina, na Região de Saúde de Lisboa*. XXIV Congresso de Pneumologia. Porto, Portugal

**Perdigão J**, Milho C, Furtado C, Carrilho L, Brum L, Portugal I. 2008. *Transmissão da tuberculose em Lisboa: Análise de uma população de estudo e caracterização por Mycobacterial Interspersed Repetitive Unit*. XXIV Congresso de Pneumologia. Porto, Portugal

**Perdigão J**, Macedo R, Silva C, Pinto C, Furtado C, Brum L, Portugal I. 2010. *Perspectiva Laboratorial da Tuberculose Resistente na Região de Saúde de Lisboa*. X & VIII Congresso Nacional de Doenças Infecciosas e Microbiologia Clínica & sobre SIDA. Coimbra, Portugal



## RESUMO

A tuberculose (TB) multirresistente (MDR) suscita ainda sérias preocupações no âmbito de saúde pública. Apesar de Portugal registar uma tendência decrescente ao nível da incidência da TB nos últimos 20 anos, dados relativos à incidência de MDR-TB aparentam sofrer de alguma subnotificação quando comparados com os dados laboratoriais existentes.

Com o presente estudo pretendeu-se obter uma nova perspectiva do estado da TB em Portugal utilizando para tal técnicas de biologia molecular aplicadas ao estudo de estirpes circulantes de MDR-TB. Pretendeu-se focar este trabalho especificamente na Região de Saúde de Lisboa. Trata-se de uma região que face aos restantes padrões europeus apresenta uma incidência de TB considerada elevada, sendo neste aspecto apenas superada pela região do Porto. Estudos prévios realizados nesta região demonstraram a existência de um conjunto de estirpes geneticamente relacionadas, o qual foi designado por Família Lisboa.

São inicialmente descritas, de entre um conjunto de 58 isolados de MDR-TB, as mutações associadas à resistência a antibacilares de primeira linha mais prevalentes: C-15T na região promotora do operão *mabA-inhA*, para a isoniazida; S531L no gene *rpoB*, para a rifampicina; M360V no gene *embB*, para o etambutol; K43R no gene *rpsL*, para a estreptomicina; e, V125G no gene *pncA*, para a pirazinamida. Do ponto de vista genotípico verificou-se uma elevada prevalência do *cluster* Lisboa3, englobando 20.7% dos isolados analisados. Foi ainda registada uma elevada taxa de TB extensivamente resistente (XDR), correspondendo a cerca de 56% dos isolados MDR estudados.

Uma análise posterior de 26 isolados clínicos XDR, quer do ponto de vista genotípico quer do ponto de vista da base molecular da resistência, mostrou a existência de uma elevada clonalidade traduzida pela distribuição da totalidade dos isolados estudados por dois *clusters* genéticos distintos: Lisboa3 e Q1. Foram ainda identificadas mutações específicas, determinantes para a base molecular da resistência e associadas com os *clusters* descritos: foram identificadas mutações nos genes *tlyA* e *rrs* exclusivamente nos *clusters* Lisboa3 e Q1, respetivamente; as mutações D94G e D94A no gene *gyrA* foram identificadas apenas nos *clusters* Lisboa3 e Q1, respetivamente; e, uma mutação no codão 91 do mesmo gene foi identificada somente em isolados do *cluster* Lisboa3. Os resultados obtidos apoiam a hipótese de aquisição múltipla de resistência a antibióticos de segunda linha com expansão clonal na comunidade. A elevada prevalência de mutações no gene *tlyA* é ainda reveladora do importante papel destas como mecanismo de resistência à capreomicina na região tornando-se importante na tomada de decisão acerca da implementação de testes rápidos de diagnóstico molecular na região.

A análise dos registos de laboratório provenientes de um período de seis anos (2001-2006), aliada à genotipagem por MIRU-VNTR, permitiu verificar o nível elevado de XDR-TB na região, oscilando esta taxa entre valores próximos ao determinado inicialmente. Foi ainda observado um aumento gradual, de ano para ano, da fracção de isolados resistentes a todos os antibióticos de primeira linha. Verificou-se igualmente, entre 2004-2006, uma predominância das estirpes pertencentes aos *clusters* Lisboa3 e Q1 como principais responsáveis pelos casos de M/XDR-TB. Estes dados apontam para uma circulação contínua deste tipo de estirpes, evidenciando, juntamente com os dados moleculares de análise mutacional, um elevado nível de endemismo da M/XDR-TB na região.

Para tentar perceber melhor a dinâmica de aquisição de resistências ao nível dos antibióticos de segunda linha, bem como a base molecular da resistência a injectáveis de segunda linha foram ainda pesquisadas mutações no gene *eis*, *tlyA*, *rrs* e *gyrA* numa amostra de 44 isolados clínicos que inclui os 26 isolados XDR-TB analisados previamente. As mutações mais prevalentes encontradas nos genes pesquisados foram: Ins755GT, A1401G, G-10A e S91P nos genes *tlyA*, *rrs*, *eis* e *gyrA*, respetivamente. Foram ainda detectadas duas estirpes isoladas nos anos 90 que apresentariam já um perfil mutacional característico de XDR-TB. Estes dados são indicativos de que a XDR-TB em Portugal seria já uma realidade nos anos 90, na altura associada ao *cluster* Lisboa3.



Uma vez que a base molecular da resistência à estreptomicina era desconhecida em cerca de 33% dos isolados MDR com resistência à estreptomicina, foi investigado o possível papel de mutações ao nível do gene *gidB*. A pesquisa de mutações neste gene revelou a ocorrência de duas mutações não-sinónimas (*missense*), das quais uma – A80P foi encontrada somente em isolados sem mutações nos genes *rrs* e *rpsL* e exclusivamente associada ao *cluster* Q1. A caracterização do nível de resistência à estreptomicina, através de um teste de susceptibilidade a antibacilares semiquantitativo, mostrou que as estirpes com a mutação *gidBA80P* apresentavam um nível de resistência intermédio à estreptomicina. Os resultados aqui obtidos apontam portanto no sentido de a mutação A80P no gene *gidB* ser responsável por um nível resistência à estreptomicina mais reduzido e ainda poder ser utilizada como marcador filogenético para o *cluster* Q1.

Uma vez estabelecidos os principais mecanismos de resistência aos fármacos de primeira linha e segunda linha e, também avaliada a clonalidade da estirpes circulantes e responsáveis pela maioria dos casos de M/XDR-TB, procurou-se caracterizar melhor estas estirpes. Para tal, foi analisado um conjunto de 56 isolados clínicos de *Mycobacterium tuberculosis*, a maioria dos quais provenientes da Região de Saúde de Lisboa. Estas estirpes foram inicialmente genotipadas pela técnica de MIRU-VNTR com 24 *loci*. Verificou-se que o número adicional de *loci* face à mesma técnica mas utilizando apenas 12 *loci*, permitiu discriminar o *cluster* Lisboa3 em dois *clusters* distintos: Lisboa3-A e -B, sendo que apenas o *cluster* Lisboa3-B estava associado a casos de XDR-TB. Foi igualmente detetada a presença do *cluster* Q1, com associação a casos de XDR-TB, em que a utilização de *loci* adicionais permitiu a discriminação de alguns isolados clínicos, presumivelmente devido a fenómenos de evolução diversificante ao nível destes *loci*.

O genoma dos 56 isolados clínicos referidos foi igualmente sequenciado na totalidade através de uma plataforma de *Next Generation Sequencing* (Illumina HiSeq 2000). Os dados genómicos resultantes permitiram verificar uma elevada predominância de isolados pertencentes ao SCG 5 e, a construção de uma filogenia baseada em SNPs, na qual foram incluídas 19 estirpes de *Mycobacterium tuberculosis* cujo genoma já havia sido previamente publicado. Do ponto de vista filogenético, pôde observar-se que as estirpes Lisboa3 e Q1 constituem dois clades monofiléticos distintos mas bastante próximos. Os dados produzidos através desta abordagem contribuem para o conhecimento acerca da diversidade genómica de *Mycobacterium tuberculosis* tendo permitido identificar mutações adquiridas no processo de microevolução associado à aquisição de resistência aos antibacilares. Foram identificadas possíveis mutações

compensatórias para a aquisição de resistência à rifampicina nos genes *rpoB* e *rpoC*, que codificam para diferentes subunidades da RNA polimerase.

Foram igualmente identificadas possíveis variantes genómicas estruturais específicas para diferentes clades filogenéticos aqui apresentados. De facto, foi identificada uma deleção de 112-3 bp no gene PPE41 nos isolados do clade Lisboa3, e é aqui proposta como um possível mecanismo de evasão imunitária, logo como uma vantagem seletiva destas estirpes. O mapeamento das sequências de inserção IS6110 comprovou igualmente o importante papel destas sequências de inserção na génese de diversidade genómica funcional. Presume-se que estas sequências de inserção tenham um papel importante na evolução do Complexo *Mycobacterium tuberculosis* uma vez que a sua transposição afeta a integridade e regulação génica.

A análise dos rácios de mutações não-sinónimas/sinónimas revelaram ainda heterogeneidades ao nível do cromossoma bacteriano, genótipo e *Clusters* de Grupos Ortólogos, realçando possíveis e diferentes estratégias de evolução. Globalmente estes resultados apoiam a noção de uma crescente diversidade genómica face a uma adaptação geográfica e ao hospedeiro.

**Palavras-chave:** *Mycobacterium*, XDR, resistência, sequenciação genómica, MIRU-VNTR, epidemiologia molecular.

## ABSTRACT

Multidrug-resistant (MDR) tuberculosis (TB) constitutes a major public health concern in Portugal. The present work was aimed at describing the MDR-TB strains circulating in Lisbon Health Region from a molecular viewpoint and, simultaneously assess its genomic diversity and phylogenetic origin.

Here, we start by describing the main mutations associated with first-line drug resistance and by revealing the presence of a high rate (56%) of extensively drug resistant (XDR) TB. Resistance to second-line drugs is further substantiated by the description of mutations found in second-line drug resistance associated genes and its association with genetic clusters, indicating clonal expansion in the community. Typing by Mycobacterial Interspersed Repetitive Unit – Variable Number of Tandem Repeats (MIRU-VNTR) revealed two major *clusters* of strains - Lisboa3 and Q1, mainly responsible for the M/XDR-TB strains in circulation. Review of laboratory data from a 6-year period confirmed the high and continued prevalence of XDR-TB strains, which was found to oscillate between 44.3-57.3% in the studied period.

Analysis of the *eis* gene promoter region identified a hypermorphic mutation (G-10A) in this region that is associated with kanamycin resistance in Lisboa3 isolates. Additionally, a mutational screening of the *gidB* gene revealed a missense mutation (A80P) that is proposed to confer intermediate-level streptomycin resistance and simultaneously act as a marker for Q1 *cluster* isolates.

Whole-genome sequencing of 56 *Mycobacterium tuberculosis* isolates allowed the construction of a genome-wide SNP-based phylogeny showing that Lisboa3 and Q1 isolates

form two distinct and unique monophyletic clades. Further genome analysis led to the identification of putative rifampicin resistance compensatory mutations and the identification of clade-specific genomic structural variants. In fact, a 112-3bp deletion found among Lisboa3 isolates is proposed to contribute towards immune evasion and virulence. *IS6110* is also addressed as a major driver in functional genomic diversification with putative impact on host adaptation.

**Keywords:** Mycobacterium, XDR, drug resistance, whole genome sequencing, MIRU-VNTR, molecular epidemiology.

## ABBREVIATIONS

3R	Replication, recombination and repair
µg	Micrograms
µl	Microlitres
aa	aminoacid
AD	<i>Anno Domini</i>
AFB	Acid Fast Bacilli
AG	Arabinogalactan
aLRT	Approximate Likelihood Ratio Test
AMK	Amikacin
ATP	Adenosine triphosphate
ATS	American Trudeau Society
BC	Before Christ
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
CAP	Capreomycin
CAS	Central Asian
CDC	Centers for Disease Control and Prevention
CFUs	Colony Forming Unit
CFZ	Clofazimine
CIP	Ciprofloxacin
COGs	Cluster of Orthologous Groups
CS	Cycloserin
CTAB	cetyl trimethylammonium bromide
DOTS	Directly Observed Short-Course Treatment

DST	Drug Susceptibility Test
<i>E. coli</i>	<i>Escherichia coli</i>
EAI	East-African-Indian
ECDC	European Centre for Disease Prevention and Control
EEA	European Economic Area
ELISA	Enzyme-Linked Immunosorbent Assay
ELISpot	Enzyme-Linked Immunospot
EMB	Ethambutol
ETH	Ethionamide
EU	European Union
EU-15	Countries in the European Union prior to 1 May 2004
FASII	Type II Fatty Acid Synthase System
FQ	Fluoroquinolone
GTR	General Time Reversible
GTX	Gatifloxacin
GWAS	Genome-Wide Association Studies
h	Hours
HGT	Horizontal Gene Transfer
HIV	Human Immunodeficiency Virus
IFN	Interferon
IGRA	Interfer Gamma Release Assay
INH	Isoniazid
INSA-RJ	National Institute of Health Dr. Ricardo Jorge
IS	Insertion Sequence
KAN	Kanamycin
Kbp	Kilo base pairs
km <sup>2</sup>	Square kilometers
L	Litre
LAM	Latin American Mediterranean
LArAM	Lipoarabinomannan
LJ	Löwenstein-Jensen
LSP	Large Sequence Polymorphism
LTBI	Latent Tuberculosis Infection
LTR	Long Terminal Repeats

<i>M. africanum</i>	<i>Mycobacterium africanum</i>
<i>M. bovis</i>	<i>Mycobacterium bovis</i>
<i>M. canetti</i>	<i>Mycobacterium canetti</i>
<i>M. microti</i>	<i>Mycobacterium microti</i>
<i>M. tuberculosis</i>	<i>Mycobacterium tuberculosis</i>
MDR	Multidrug Resistant/Multidrug Resistance
mg	Miligrams
MGIT	Mycobacterial Indicator Growth Tube
MIC	Minimum Inhibitory Concentration
min	Minutes
MIRU	Mycobacterial Interspersed Repetitive Unit
ml	Mililitres
MOX	Moxifloxacin
MTC	<i>Mycobacterium tuberculosis</i> Complex
NAAT	Nucleic Acid Amplification Test
NAD	Nicotinamide Adenine Dinucleotide
ng	Nanograms
NGS	Next-Generation Sequencing
N <sub>s</sub>	Non-synonymous
nsSNP	Non-synonymous Single Nucleotide Polymorphism
nt	Nucleotide
°C	degrees Celsius
OFX	Ofloxacin
ORF	Open Reading Frame
PAS	Para-mino Salicylic Acid
PCR	Polymerase Chain Reaction
PGG	Principal Genetic Group
PGM	Personal Genome Machine
PHA	Portuguese Health Authorities
pmol	Picomoles
POA	Pyrazinoic Acid
PPD	Purified Protein Derivative
PTH	Prothionamide
PZA	Pyrazinamide

Pzase	Pyrazinamidase
QBP	Quinolone Binding Pocket
qDST	semiquantitative Drug Susceptibility Testing
QFT-GIT	QuantiFERON®-TB Gold In-tube
RD	Region of Difference
REMA	Resazurin Microplate Assay
RFLP	Restriction Fragment Length Polymorphism
RIF	Rifampicin
RNA	Ribonucleic Acid
RRDR	Rifampicin Resistance Determining Region
rRNA	Ribosomal Ribonucleic Acid
S	Synonymous
SCG	SNP Cluster Group
sec	Seconds
SNP	Single Nucleotide Polymorphism
sSNP	Synonymous Single Nucleotide Polymorphism
STP	Streptomycin
SV	Structural Variant
TB	Tuberculosis
TNF	Tumor Necrosis Factor
T <sub>s</sub>	Transition
T-SPOT	T-SPOT®.TB
TST	Tuberculin Skin Test
T <sub>v</sub>	Transversion
UPGMA	Unweighted Pair Group Method with Arithmetic Averages
VCF	Variant Call Format
VIO	Viomycin
VNTR	Variable Number of Tandem Repeats
vs	<i>versus</i>
WGS	Whole-Genome Sequencing
WHO	World Health Organization
XDR	Extensive Drug Resistant/Extensive Drug Resistance



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# CHAPTER 1

## General Introduction





## 1.1 BRIEF HISTORIC PERSPECTIVE

It is assumed that tuberculosis (TB) and mankind coexist since pre-historic times, having co-evolved through time. Human beings have emerged in Africa and formed small hunter-gatherer family groups, composed by one or two dozens of individuals. However, their dialectic groups could comprise a few hundred individuals and, numerous groups such as these could have had the capacity to withstand TB epidemics. When these ancestral humans migrated out of Africa, and even before settling in stable communities, they took this disease with them as they peopled the world. Afterwards, TB epidemic explosions took place as sedentary societies were settled.<sup>1, 2</sup>

The way human TB has appeared has been surrounded by some controversy, since the first theories stated that ancestral human TB would have emerged due to cattle domestication, leading to the contagion of ancestral human beings with the bovine tubercle bacilli. According to this theory, the modern human tubercle bacilli would have evolved from the bovine tubercle bacilli.<sup>1</sup> However, and since no domesticated cattle existed in North America until the arrival of the Spanish in the 15th century, it is more likely that human and bovine tubercle bacilli have evolved from a common ancestral. Recent molecular data point towards this latter hypothesis as the most likely.<sup>3, 4</sup>

The oldest Neolithic graves with TB signs date back to 5000 BC and 4000 BC, discovered near Heidelberg, Germany, and Liguria, Italy.<sup>5</sup> Both graves contained mortal remains evidencing the TB characteristic vertebral destruction. It is however in Ancient Egypt that the greatest number of mummified mortal remains, with vertebral lesions indicating TB, have been retrieved. Posterior analyses have detected the presence of DNA from the bacterial complex responsible for this disease (*Mycobacterium tuberculosis* Complex, MTC) in several mummies from a 3000 year time period (3400-500 BC).<sup>6-10</sup> In a study by Zink *et al*, MTC DNA was detected in 25 out of 85 Egyptian mummies, of which it was possible to genotype 12 by spoligotyping and exclude the bovine tubercle bacilli as the cause of the disease.<sup>11</sup>

In the American continent, TB was already present well before the arrival of Columbus in the 15th century. Mummies found in Peru (700 and 1000 AD) showed the same vertebral lesions characteristic of this form of the disease and, in one case MTC DNA was detected.<sup>12, 13</sup> Furthermore, several skeletons from American Indian graves were found with TB signs and there are examples of Mayan artwork and drawings that portray TB symptoms.<sup>1</sup>

Tuberculosis was also present in the pre-historic Asia and several references to a similar disease in India and China between 2000 and 1300 BC exist, with commentaries regarding symptomatology and disease characteristics already mentioning it as incurable.<sup>1, 14</sup>

There are Assyrian terracotta plates from the 7th century BC that describe patients coughing blood. The disease has been described by Hippocrates in the 4<sup>th</sup> century BC, as occurring mainly in individuals aged between 18 and 35 years old and used the term *phthisis*.<sup>1, 15</sup> Later, it was Galen that described TB in greater detail, suggesting that it was an infectious disease and even trying different treatments without any success.<sup>1</sup>

With the demographic explosion that took place in Europe during the second millennium AD and the enormous urban centre growth, Europe became the epicentre of innumerable epidemics, namely those that started in the 16<sup>th</sup> and 17<sup>th</sup> centuries.<sup>15</sup>

Between the 17th century, and until later in the 19th century, TB receives the name of white plague. The period that has registered the largest number of cases occurred in the second half of the 18<sup>th</sup> century, at a time that is estimated that 1.25% of the Europeans died of TB every year. England alone had an annual mortality rate of 1120 cases per 100 000 habitants due to TB. Another study from the early 19<sup>th</sup> century performed at the Hôpital de Charité in Paris, revealed that from 696 examined corpses, 250 would have died of TB.<sup>1, 15</sup>

In the second half of the 19th century the TB associated mortality rate started to decline as sanitation and housing conditions improved (*e.g.* Paris urban renewal).

The cause for TB remained, nevertheless, unknown for centuries. In Northern Europe it was generally believed that it was a hereditary disease, while in Southern Europe it was regarded as an infectious disease. A first step in demonstrating that it was in fact an infectious disease was taken by Jean Antoine de Villemain, in 1865, by inoculating rabbits with human pulmonary extracts from tuberculous lesions and inducing similar lesions. However, only in 1882 it was definitely demonstrated by Robert Koch that TB was in fact an infectious disease, whose etiological agent is a bacteria, when he isolated and identified *Mycobacterium tuberculosis* (*M. tuberculosis*).<sup>15, 16</sup>

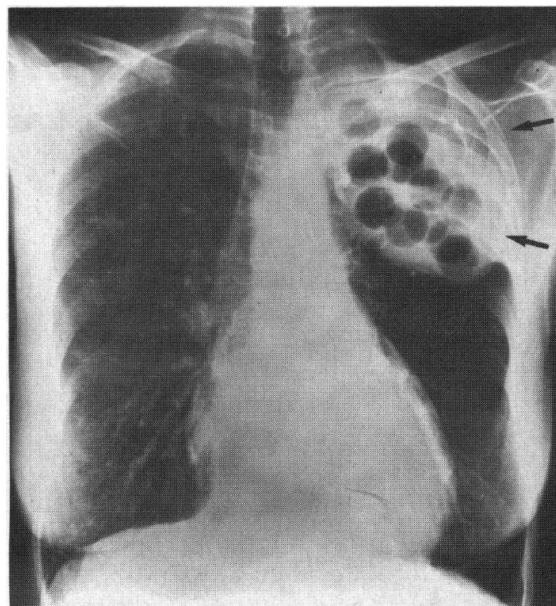
Still in late 19<sup>th</sup> and early 20<sup>th</sup> centuries, an experience conducted by Edward Trudeau showed that TB could be induced in rabbits using virulent cultures, with a variable prognosis depending on the environmental conditions in which the animals were kept in. This experience has greatly contributed to promote the sanatoria movement that had already started in Europe in late 19<sup>th</sup> century (Figure 1.1). The sanatoria were places in which patients were committed for several

months or years, with a treatment based on resting, fresh air and a balanced diet. Although today is still doubtful if this treatment improved the patient survival rate, the fact that these stayed isolated from the rest of the community may have effectively contributed for the reduction of the number of cases.<sup>1, 17</sup>



**Figure 1.1** - Patients resting in reclining chairs in the BASF Sanatorium (1892) in Dannenfelds, Germany  
Extracted from Campbell.<sup>18</sup>

In early 20th century, some surgical techniques started to be used in TB treatment. Many of these techniques relied on the principle that the lung would need to rest in order to heal the lesions. Techniques such as the artificial pneumothorax that induced lung collapse and allowed the lung to rest started to be employed. More drastic were the thoracoplasty (surgical removal of ribs) or extra pleural plombage (paraffin infection or other inert material in the pleural cavity) that induced a permanent pulmonary collapse (Figure 1.2). Another procedure would consist in the unilateral crushing of the phrenic nerve, which would stop the diaphragm activity, also with the purpose of lung resting. Although for many patients, the collapse therapies may have saved their lives it showed very high risks and complications in the long-term.<sup>1, 17</sup>



**Figure 1.2** – Posteroanterior chest x-ray film from a 64-year old woman treated for tuberculosis by stripping the parietal pleura from the chest wall and packing the space with inert Lucite balls (arrows). Extracted from Mond & Khan.<sup>19</sup>

In the 1920s, Albert Calmette and Camille Guérin produce the first vaccine for TB prophylaxis, named *Bacillus Calmette-Guérin* (BCG), from the sequential passage of a virulent *Mycobacterium bovis* (*M. bovis*) strain. Later, in the 40s the era of TB antibiotic treatment starts with Albert Schatz and Selman Waksman and the discovery of streptomycin (STP). In the

following years the main antibacillary drugs still used today to fight TB are (re)discovered, *e.g.*, isoniazid (INH), rifampicin (RIF) and pyrazinamide (PZA).<sup>1, 17</sup>

The implementation of adequate treatment regimens with effective antibacillary drugs has lead to an accentuated reduction in the number of cases in many countries during the 60s and 70s, which also lead to the belief that the fight against TB was won. Nevertheless, the combined emergence of resistant strains and the human immunodeficiency virus (HIV) are still today threatening the control of this disease.<sup>1, 17</sup>

## **1.2 TUBERCULOSIS IN THE PRESENT**

### **1.2.1 Tuberculosis in the World**

The worldwide number of TB cases still reaches alarming proportions. Although at global level the disease incidence and prevalence present a decreasing trend in last 10 and 15 years, respectively, the most recent report from the World Health Organization (WHO) point out to an estimated occurrence of 8.7 million new cases in 2011 (Table 1.1). This number of incident cases is reflected by a high global incidence rate of 125 cases per 100 000 habitants. The most affected regions are Asia and Africa, with 59 and 26% of the total number of new cases worldwide. On the other hand, countries like India in which the total number of new cases (2.2 million) corresponds to 26% of the total number of new cases worldwide, simultaneously show a comparatively lower incidence rate (181 cases per 100 000 habitants). Other countries, such as South Africa and Zimbabwe, due to the high HIV co-infection rates have incidence rates of 993 and 603 new cases per 100 000 habitants, respectively.<sup>20</sup>

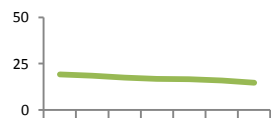
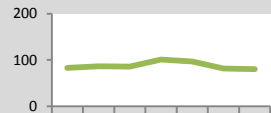
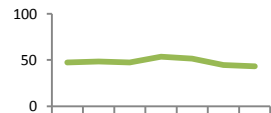
Regarding mortality due to TB, WHO estimates that 1.4 milion deaths have occurred in 2011 due to TB, of which 0.43 million took place among HIV-positive individuals. Although number of deaths stratified by region was only reported by the WHO for HIV-negative patients: in absolute numbers, it was verified a higher number of mortal cases in the Southeast Asia region with an estimate of 480 000 deaths. Mortality rate was higher in the African and Southeast Asia regions, both with a mortality rate of 26 deaths per 100 000 habitants (Table 1.1).<sup>20</sup>

**Table 1.1** – Incidence, prevalence, mortality and HIV coinfection rate across the different WHO Regions in 2011. Data extracted from the WHO surveillance report.<sup>20</sup>

WHO Region	Incidence		Prevalence		Mortality <sup>a</sup>		HIV
	thousands	per 100 000 Hab.	thousands	per 100 000 Hab.	thousands	per 100 000 Hab.	%
Africa	2 300	262	2 500	293	220	26	39.0
Americas	260	28	330	35	21	2.2	14.0
Eastern Mediterranean	660	109	1000	170	99	16	1.5
Europe	380	42	500	56	45	5.0	6.1
Southeast Asia	3 500	189	5000	271	480	26	4.1
Western Pacific	1 700	92	2500	138	130	6.9	2.2
<b>Global</b>	<b>8 700</b>	<b>125</b>	<b>12 000</b>	<b>170</b>	<b>990</b>	<b>15</b>	<b>13</b>

<sup>a</sup>WHO Mortality indicator due to TB excludes deaths among HIV-positive patients

**Table 1.2** – TB notification rates (per 100 000 habitants) and tendency across the European Region between 2004-2010. Data extracted from the European Centre for Disease Prevention and Control/WHO Regional Office for Europe surveillance report.<sup>21</sup>

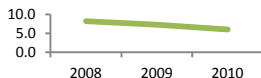
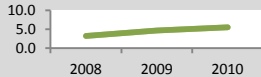
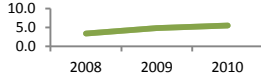
Countries	2004	2005	2006	2007	2008	2009	2010	Trend
<b>EU/EEA</b>	19.1	18.5	17.5	16.8	16.5	15.8	14.6	
<b>Non-EU/EEA</b>	82.8	86.5	85.5	100.6	96.7	81.3	80.1	
<b>Europe</b>	47.3	48.2	47.3	53.4	51.6	44.4	43.2	

In Europe, according to the European Centre for Disease Prevention and Control (ECDC), 388 875 new cases were notified in 2010 resulting in an apparent decline of 15.2% in the period of 2005-2010. Notification rates registered for each country in this region ranged between 2.8 and 123 cases per 100 000 habitants in Italy and Kazakhstan, respectively. In 2010, the global notification rate for the European region was 43.2 cases per 100 000 habitants, although it was significantly lower in the European Union (EU) and European Economic Area (EEA) countries than in the other countries group: 14.6 vs 80.1 cases per 100 000 habitants, respectively (Table

1.2). Both country groups have presented a decreasing trend in the notification rates, more accentuated in the EU/EEA countries, -4.4% in the 2006-2010 period, when compared with the other countries in the same period (-0.9%) (Table 1.2).<sup>21</sup>

The HIV/TB co-infection rate globally registered in Europe was 5.5% in 2010, with a growing tendency in the last 3 years. This tendency is the result of diverging tendencies registered among the groups of EU/EEA and other countries. In the EU/EEA countries a decreasing tendency in the number of TB/HIV co-infected cases has been registered in the last 3 years, while the remaining countries have registered an increase in the TB/HIV co-infection rate in the same period that results in the increasing global European co-infection rate (Table 1.3).<sup>21</sup>

**Table 1.3** – Number, percentage and tendency of TB/HIV co-infection between 2008-2010. Data extracted from the European Centre for Disease Prevention and Control/WHO Regional Office for Europe surveillance report.<sup>21</sup>

Countries	2008		2009		2010		Trend (%)
	N	(%)	N	(%)	N	(%)	
<b>EU/EEA</b>	1402	8.2	1334	7.3	1055	6.0	
<b>Non-EU/EEA</b>	10397	3.2	12187	4.6	14779	5.5	
<b>Europe</b>	11799	3.4	13521	4.8	15834	5.5	

With the exception of Ukraine, all countries that have a TB/HIV co-infection rate above 10% belong to EU/EEA. The six countries with the highest TB/HIV co-infection rates are: Ireland (17.6%), Portugal (13.3%), Ukraine (13.0%), Netherlands (12.7%), Estonia (11.5%) and Malta (11.5%).<sup>21</sup>

Concerning mortality in Europe due to TB, the ECDC estimates that 60 135 deaths occurred due to TB (6.7 per 100 000 habitants) in 2010 in the WHO European space. About 91% of these mortal cases occurred in countries other than the ones in the EU/EEA (54 752 deaths, 140 deaths per 100 000 habitants), in comparison with the countries that integrate this latter group (5 383 deaths, 1.1 deaths per 100 000 habitants).<sup>21</sup>

### 1.2.2 Tuberculosis in Portugal

Portugal is the western European country with the highest incidence rate with 2 626 cases in 2010 of which 2 398 are new cases, *i.e.*, with no previous treatment. This incidence results in a notification rate of 24.7 cases per 100 000 habitants or 22.5 cases per 100 000 habitants if only the new cases are considered.<sup>21</sup> This incidence rate can be classified as intermediate by comparison with the remaining European countries. According to the Portuguese Health Authorities (PHA) this notification rate has a decreasing tendency for the last 19 years with a mean annual reduction of 6.4%.<sup>22</sup>

The geographical distribution of the cases at a nationwide level show a higher case incidence in the districts of Lisbon and Oporto, the only districts with notification rates above 30 cases per 100 000 habitants. Lisbon is according to the PHA data for 2010 the district with highest notification rate (31.5 cases per 100 000 habitants) and absolute number of cases (706 new cases).<sup>22</sup> Six districts, however, still present notification rates with values comprehended between 20-30 cases per 100 000 habitants: Viana do Castelo, Vila Real, Bragança, Setúbal and Faro. Provisory PHA data show that in 2011 the Oporto district had the highest TB notification rate (31.9 cases per 100 000 habitants).<sup>23</sup>

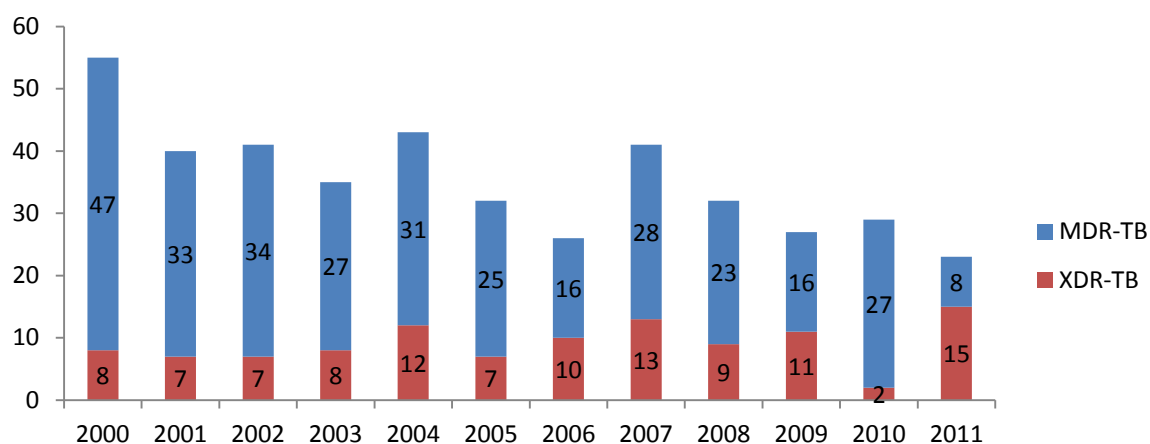
At a demographic level, a higher incidence of the disease was observed in male individuals at a 1.9:1 ratio and, a median age shift from the 25-34 age group to the 35-44 age group, occurred in the last decade.<sup>22</sup>

The number of cases observed among immigrants was 397 in 2010, 16% of the total notified cases. The origin of the majority of these foreign individuals was: Angola (23%), Guinea-Bissau (16%), Cape Verde (13%), Brazil (12%) and Mozambique (9%).<sup>22</sup> The 2011 PHA provisory data, showed 372 new cases among immigrants were observed with a significant increase in patients from Cape Verde (18%) and from Romania (7%).<sup>23</sup>

According to the ECDC, concerning drug resistant cases, 68 INH resistant and 19 RIF resistant cases were notified in 2010, with these latter also exhibiting INH resistant, *i.e.*, multidrug resistant (MDR).<sup>21</sup> In 2010, no case of extensively drug resistant (XDR) TB (multidrug resistance plus resistance to a fluoroquinolone (FQ) and an injectable second-line drug: kanamycin (KAN), amikacin (AMK) or capreomycin (CAP)) was notified.<sup>21</sup> PHA data, slightly differ from the ECDC data since the latter reports 29 cases of MDR-TB and 2 cases of XDR-TB nationwide (Figure 1.3).<sup>22, 23</sup> These numbers appear to suffer from some degree of under notification since they

are low when compared to the number of clinical isolates reported in peer-reviewed scientific publications in the last 15 years.<sup>24-26</sup> Also important to stress is that in the ECDC reported data only 61.1% of the notified cases are laboratory-confirmed and from these, drug susceptibility testing data was available for only 76.3% in a total of 1 225 cases.<sup>21</sup> For 2011, data from PHA show a marked increase in the number of XDR-TB cases.<sup>23</sup>

The indicators for treatment success for the 2009 cohort, 24 months after diagnosis, show a success rate of 84.2%.<sup>22</sup> For this cohort the following was observed: 88 deaths (5.6%), 48 treatment defaults (3.1%), 79 patients still on treatment after 24 months (5.0%) and 32 patients lost to follow-up (2.0%).<sup>21</sup> Analysis of the 2010 cohort show a reduction in the success rate to 77%.<sup>23</sup>



**Figure 1.3** – Number of new MDR- and XDR-TB cases reported by PHA between 2000-2011. Data extracted from PHA reports.<sup>22, 23</sup>

### 1.3 MYCOBACTERIA

#### 1.3.1 General Morphological Properties

Mycobacteria are slightly curved or straight bacilli, whose dimensions range between 0.2-0.6 x 1.0-10.0 µm, although some degree of pleomorphism may be exhibited. These are aerobic microorganisms that may display a ramified growth or a filamentous growth similar to a mycelium. Contrary to the remaining Actinomycetes, a true mycelium is not formed and when subjected to some physical disturbance, fragmentation in bacilli or cocci occurs.<sup>27</sup> Nevertheless, *Mycobacterium farcinogenes* and *Mycobacterium senegalense* do form a stable mycelium.<sup>28</sup> Although controversial, recent studies performed with *Mycobacterium marinum*



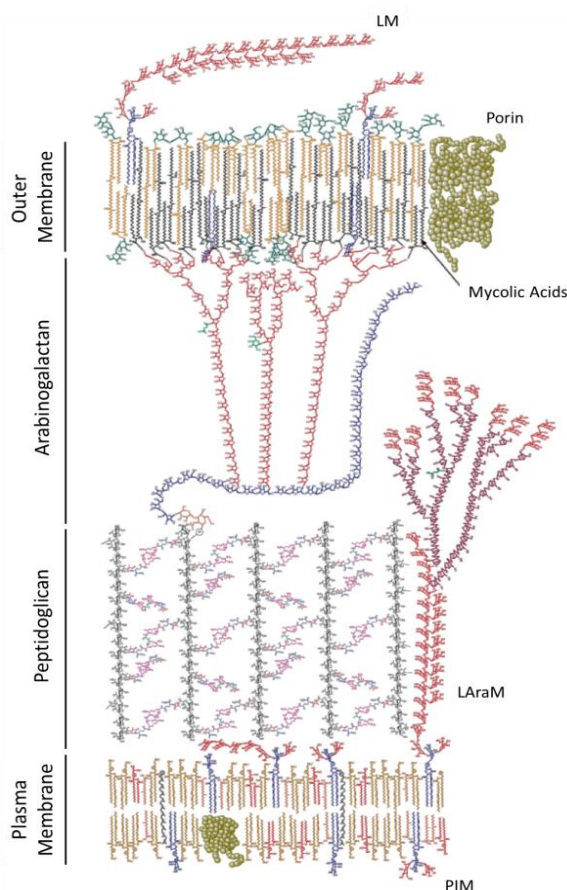
and *Mycobacterium avium* Subsp. Paratuberculosis seem to demonstrate the ability of some mycobacteria to produce endospores as a possible mechanism of persistence in the host.<sup>29-32</sup>

Regarding the colonial morphology, mycobacteria may present more than one type of colony, even varying with the species' strain. Usually, *M. tuberculosis* strains present rough colonies while the colonies of *Mycobacterium intracellulare* are usually smooth. In other species the colony morphology depends on the growth phase.<sup>28</sup>

Mycobacteria may be strictly parasitic, saprophytes or exhibit an intermediate lifestyle. Most species free lives in the water or soil, but for many species, their ecologic niche are the tissues of homeothermic hosts, e.g. *M. tuberculosis*, whose natural reservoir is the human population.<sup>28</sup>

Mycobacterial cell wall has several particularities making it significantly different from the typical Gram positive and negative cell wall types. The mycobacterial peptidoglycan consists in glycosidic alternate chains composed by N-acetylglucosamine and muramic acid binded by a  $\beta(1\rightarrow4)$  bond. The tetrapeptid, essential for cross-linking with other glycosidic chains is bound to the muramic acid. This tetrapeptid consists in L-alanil-D-isoglutaminil-meso-diaminopelil-D-alanin with an amide on the diaminopimelic acid residue. The muramic acid is acylated with a glycolyl residue instead of an acetyl residue and, the number of cross-links between the peptidoglycan chains are higher than in the peptidoglycan from *Escherichia coli*.<sup>34</sup>

Another molecule, the arabionogalactans (AG), are covalently bound to the peptidoglycan. These molecules have unusual monosaccharides and bonds and, are composed by D-arabino-furanosyl and D-galacto-furanosyl.<sup>34</sup> The AGs are esterified by mycolic



**Figure 1.4** – Theoretical model of the *M. tuberculosis* cell envelope (composed by the plasma membrane, cell wall core and outer membrane) chemical structure showing its major components. Abbreviated molecules: LArM, lipoarabinomannan; LM, lipomannan; PIM, phosphatidyl-*myo*-inositol mannosides. Adapted from Kaur *et al.*<sup>33</sup>

acids, of great importance in the taxonomical classification of mycobacteria, which are long-chained  $\alpha$ -ramified- $\beta$ -hydroxylated fatty acids that may contain up to 90 carbon atoms.<sup>28, 34</sup> The presence of mycolic acids confers on mycobacteria the property of acid-fastness, at least during some stage of its life cycle. Although mycobacteria are not stainable by Gram staining method, due to the lipidic content on the surface, if the lipidic portion is removed with alkaline ethanol, the reminescent cell becomes gram-positive.<sup>35</sup>

A complex mixture of proteins and polysaccharides has also been described around *M. tuberculosis in vitro* when non-mechanically disturbed. This structure is possibly a pseudocapsule since it is not covalently bound to the cell wall and is composed by polysaccharides such as glucans, arabinomanans, manans and proteins. Part of the proteins that compose this pseudocapsule appear to be proteins that are being transported to the exterior while others are probably permanent proteins. Besides being present in culture filtrates, these proteins are also present in considerable quantities in cell extracts obtained by mechanical treatment.<sup>34, 36</sup>

The generation time is highly variable from species to species, oscillating between 2-20 hours, which implies that colony formation may take from 2 days to 8 weeks when incubation is performed at the optimal temperature. The optimal temperature also varies according to the species, between 30-45°C.<sup>28</sup>

Culture may be performed in simple media with mineral salts, using ammonia or amino acids as nitrogen source and glycerol as a carbon source. There are, nevertheless, species that require supplementation such as hemin, micobactins or other iron transporter.<sup>28</sup>

Many mycobacteria produce yellow or orange carotenoid pigments, whose induction may or not occur by photo induction. The presence of these carotenoids may protect against oxidative damage by oxygen in the singlet form.<sup>27</sup>

Mycobacteria also have a high GC content (>50%), that may range between 62-70%.<sup>28</sup>

### 1.3.2 Mycobacterial taxonomy

Although mycobacteria were one of the early described bacterial groups, a functional taxonomy was only elaborated in the last 50 years. Mycobacteria are included in the *Mycobacteriaceae* family, that contains a single genus – *Mycobacterium*, with a present

number of 156 species and 13 subspecies.<sup>37, 38</sup> This number has increased significantly in the last two decades.<sup>39, 40</sup>

Phylogenetically close are the *Corynebacterium*, *Nocardia* and *Rhodococcus* genera, which is reflected by their high GC content and, still, by the fact that these also produce mycolic acids, although mycobacterial mycolic acids contain a higher number of carbon atoms.

A natural division exists between fast-growth and slow-growth mycobacteria. Fast-growth mycobacteria take less than seven days to develop colonies from a diluted inoculum, while fast-growers take more than seven days to form visible colonies.

Tsukamura has proposed, in 1967, the separation of fast-growers and slow-growers in two distinct sub-genera. This proposal was not adopted since both groups demonstrate a considerable degree of similarity regarding DNA homology, antigenic and lipidic composition, and antibiotic susceptibility.<sup>28</sup>

Other mycobacterial classification in three groups can be made based on pigmentation:

- Non chromogenic – do not exhibit pigment production, *e.g.*, *M. tuberculosis* or *M. bovis*;
- Photochromogenic – produce pigments through photo induction, *e.g.*, *M. marinum* e *M. kansasii*;
- Scotocromogenic – produce pigments in the absence of light, *e.g.*, *M. goodii*.

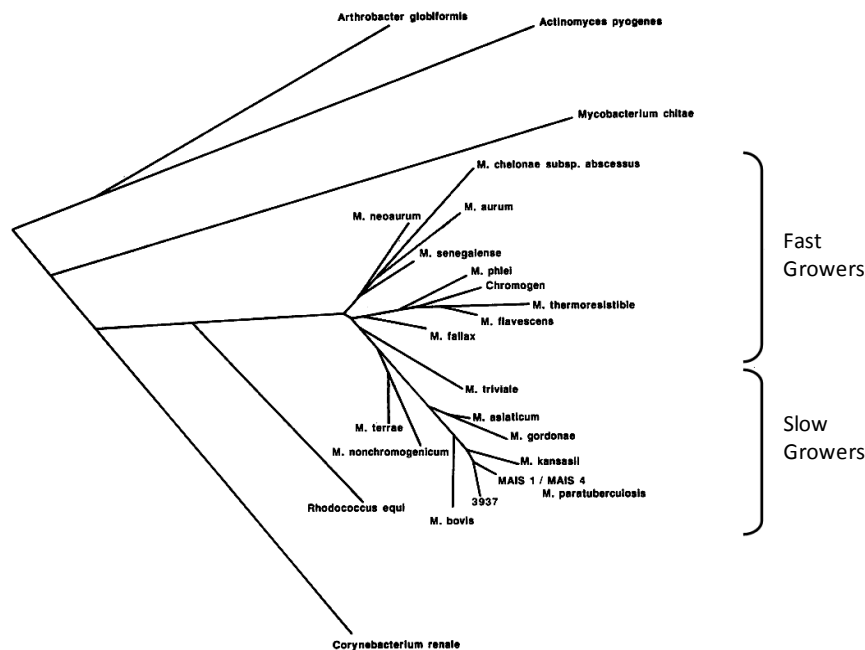
In 1959, Runyon proposed the classification of mycobacteria in 4 groups, based on pigmentation and growth rate:

- Group I – photochromogenic slow-growth mycobacteria;
- Group II – scotocromogenic slow-growth mycobacteria;
- Group III – non-cromogenic slow-growth mycobacteria;
- Group IV – fast-growth mycobacteria.

Although this is a phenotypic-based classification and does not reflect phylogenetic proximity, the Runyon classification is still used today.<sup>41</sup>

Stahl *et al* have verified through 16S rRNA comparison that the inferred phylogeny reflects the division between slow-growth and fast-growth mycobacteria, with a greater coherence between the slow-growers as these formed a distinct line of evolutionary descent (Figure

1.5).<sup>42</sup> It is however difficult to discriminate all mycobacteria at the species level since closer species can have a 16S rRNA similarity greater than 97%, the generally accepted limit for the inclusion of strains in the species taxon.<sup>27, 43</sup>



**Figure 1.5** – 16S rRNA-based phylogeny of several mycobacterial species and related microorganisms. Adapted from Stahl *et al.*<sup>42</sup>

More recent multigenic studies, involving not only the nucleotide sequence of the 16S rRNA but also nucleotide and amino acid sequences of additional genes (*e.g.*, *hsp65* and *rpoB*) have allowed a better discrimination of mycobacterial species and confirm the same phylogenetic division and monophyletic origin of slow-growth mycobacteria.<sup>43-45</sup> According to this phylogeny, the slow-growers' group has probably evolved from one of the two main fast-growth mycobacterial groups.<sup>44, 45</sup>

### 1.3.3 *Mycobacterium tuberculosis* complex

TB-causing mycobacteria form a group designated by *Mycobacterium tuberculosis* complex (MTC), that includes *M. tuberculosis*, *M. bovis* (subspecies *bovis* and BCG), *Mycobacterium africanum* (*M. africanum*, subtypes I and II), *Mycobacterium microti* (*M. microti*) and *Mycobacterium canetti* (*M. canetti*, not officially recognized). The MTC species can be considered as ecotypes of the same species, adapted to different hosts. Nevertheless, since the MTC species have been formally described, the above nomenclature is still used.

In 2003, Cousins *et al* have proposed the integration of a new member in this complex, *Mycobacterium pinnipedii*, that has already been characterized and integrated in the MTC.<sup>46</sup> In the same year, it was equally recognized as belonging to the MTC the *Mycobacterium caprae* species, initially described as a *M. tuberculosis* subspecies and later as a *M. bovis* subspecies.<sup>47-</sup>

<sup>49</sup> More recently, two new species were proposed to be added to the MTC, *Mycobacterium mungi* and *Mycobacterium orygis*.<sup>50, 51</sup>

Genetically, all members of this complex are quite similar, having about 99.9% of nucleotide sequence similarity and identical 16S rRNA sequences.

MTC species can be differentiated by phylogenetic-informative genomic deletions that seem to demonstrate that *M. microti*, *M. bovis* and *M. africanum* have a more recent common ancestor than with *M. tuberculosis*. This phylogeny suggests that the ancestral species of *M. tuberculosis* has diverged, which was followed by speciation events that have led to the origin of the remaining MTC species, with the exception of *M. canetti*. Brosch *et al* also proposed that the common ancestor for the entire MTC would be similar to *M. tuberculosis* or *M. canetti* (the first species to diverge) and would already be a human pathogen.<sup>3</sup>

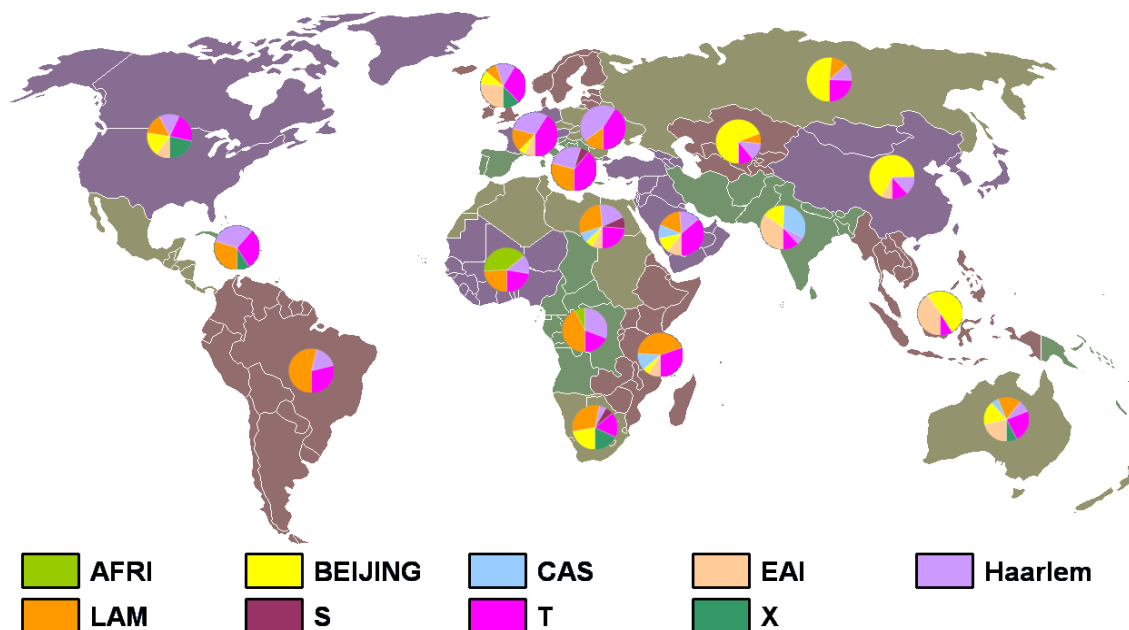
#### 1.3.4 Genetic Diversity, Evolution and Adaptation

For several years, the *M. tuberculosis* species has been regarded as a highly specific adaptation to the human species, considered to be its only reservoir, possibly derived from another species with a broader host variety, such as *M. bovis*. Sreevatsan *et al* have classified *M. tuberculosis* as a recent species and proposed that the speciation event would have occurred between 25 000 – 30 000 years ago. According to these authors, the fact that it was a fairly recent species would explain the reduced genomic variability found when characterizing 26 structural genes from clinical isolates.<sup>52</sup>

More recent comparative genomic analysis has led to the conclusion that the between-lineage existing genetic diversity is higher than the one initially estimated. Gutierrez *et al* when comparing the accumulated differences in six structural genes of 17 strains have estimated that the speciation would have occurred between 2.6 – 2.8 million years ago.<sup>53</sup> This estimate is therefore higher than the proposed by Sreevatsan *et al* and places *M. tuberculosis* as one of the most ancient pathogens, that has inclusively affected the early hominids.<sup>52, 53</sup>

During the last two decades several techniques have been employed in the assessment of *M. tuberculosis* diversity, some with a high discriminatory power and, hence, of enormous utility in molecular epidemiology through the identification of outbreaks at a regional level (e.g., RFLP-IS6110 and MIRU-VNTR). Other techniques, such as single nucleotide polymorphism (SNP) or deletion typing, have a lower discriminatory power but, are able to position a given strain in a species' global population structure.

Spoligotyping, which is discussed ahead as a molecular epidemiologic technique (see Molecular Epidemiology section) is also extremely useful and one of the most widely diffused techniques to assess global strain diversity. Global data from the international spoligotype database (SITVIT) has allowed the definition of the major lineages within *M. tuberculosis* sensu stricto: Beijing, Central Asian (CAS), East-African-Indian (EAI), Haarlem, Latin-American-Mediterranean (LAM), S, T and X (Figure 1.6). Furthermore, a total of 62 MTC sub-lineages can be defined in the SpolDB4 version.<sup>54-59</sup> The latest version of SITVIT (SITVIT1) houses spoligotyping data from 62 582 clinical isolates and defines new rules for clade assignment.<sup>60</sup>

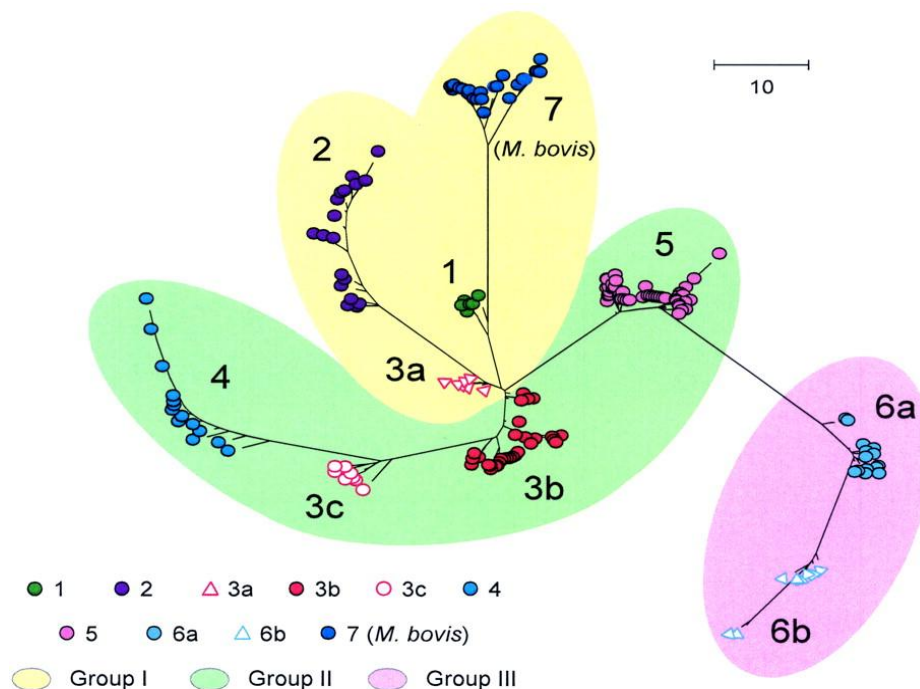


**Figure 1.6** – Prevalence and distribution of the major spoligotype lineages. Extracted from the SITVITWEB database website.<sup>60</sup>

The development of further markers was greatly boosted by the knowledge of *M. tuberculosis* genome. This first genomic analysis was initially carried out by sequencing the genome of *M.*

*tuberculosis* H37Rv, a laboratory strain derived from a clinical isolate dated back to 1905, that has maintained its virulence on animal models.<sup>61, 62</sup> Presently, several genomes of *M. tuberculosis* clinical isolates have been made available, showing an average genomic length of 4.4 Mb, with a GC content averaging 65.4%, with around 4081 genes coding for 3970 proteins.

The genomic comparison of clinical isolates from different origins with the genomic sequence of *M. tuberculosis* H37Rv has allowed the identification of a considerable number of SNPs and the construction of a global phylogeny of different *M. tuberculosis* lineages. Four studies have tried to define the *M. tuberculosis* populational structure, with the proposed phylogenies presenting a high degree of congruence.<sup>62-65</sup> Sreevatsan *et al* when analysing 26 structural genes have identified two non-synonymous SNPs (nsSNPs) that have allowed the definition of three *M. tuberculosis* Principal Genetic Groups (PGG).<sup>52</sup> This division is also congruent with other proposed phylogenies based on higher numbers of SNPs. Filliol *et al* have analysed 212 isolates, by comparison of 159 synonymous SNPs (sSNPs), and defined six SNP cluster groups (SCG) and five sub-groups (Figure 1.7). Furthermore, this phylogenetic classification has allowed to define a restrict number of six SNPs that allows the classification in one of the SCGs.<sup>64</sup> Alland *et al*, on the other hand, defined a different set of nine SNPs that allows not only the SCG classification of a given strain, but also the sub-group identification.<sup>66</sup>



**Figure 1.7** – Global phylogenetic tree of *M. tuberculosis* isolates. SCGs and its subgroups are highlighted with the respective designation and colour. PGGs are also highlighted by background colouring according with Sreevatsan *et al*. Scale bar indicates the phylogenetic distance in SNP number. Extracted from Filliol *et al*.<sup>64</sup>

Molecular data has shown that particular groups of strains with unknown selective advantages are the cause of MDR-TB outbreaks. The Beijing/W strains or the *M. bovis* B strains, in Spain, are good examples.<sup>67-70</sup> Their vast distribution reflects their success, and several studies have demonstrated that the Beijing/W strains induce a more severe pathology, suggesting an increased virulence.<sup>71</sup>

Phylogenetic classification is however insufficient for explaining possible selective advantages that might be behind high prevalence rates by some lineages or families between different human populations. However, other genetic alterations occurring in these lineages, with marked impact on strain physiology and adaptability, might be responsible for these differential prevalence rates.

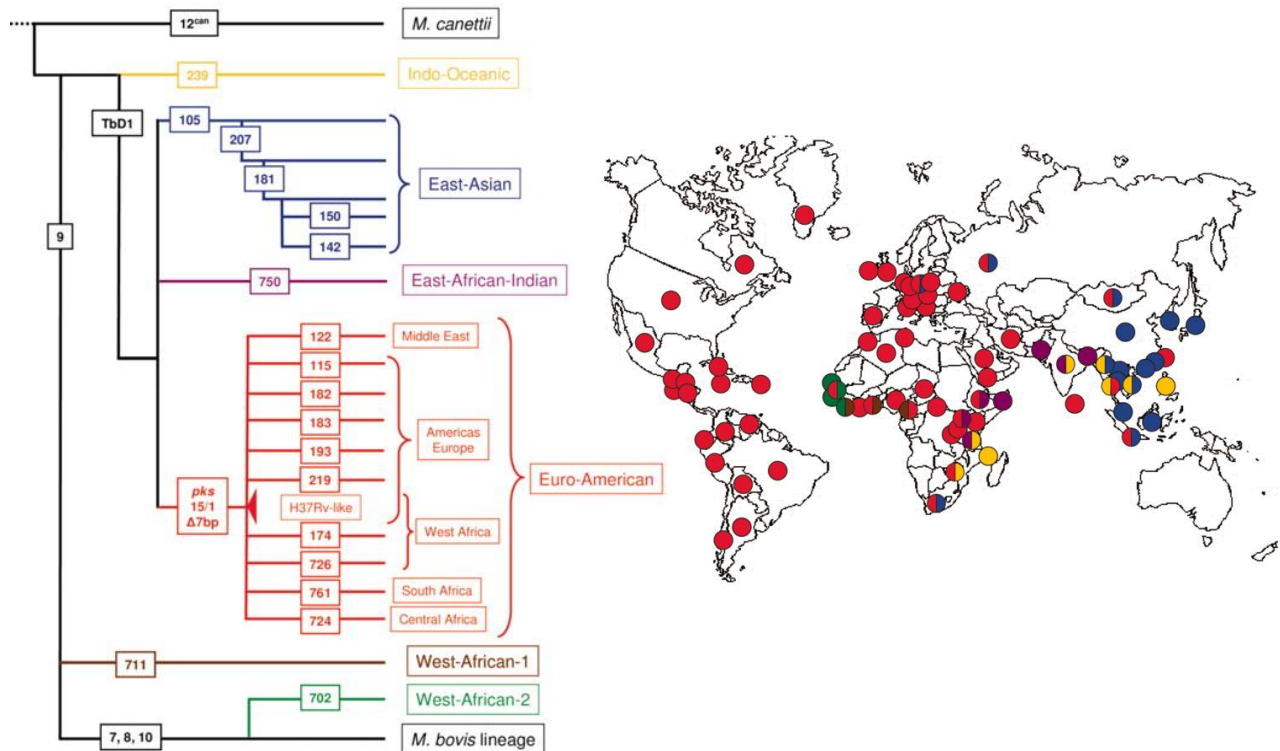
Hershberg *et al* have concluded, in a study involving a collection of globally representative isolates of *M. tuberculosis* and other MTC members, that the MTC species are under a reduced purifying selection; MTC diversity is associated with human demographic and migratory events; and, that functional diversification is lead by genetic drift.<sup>2</sup>

Horizontal Gene Transfer (HGT) is a major driver in bacterial evolution and is thought to have occurred in the MTC ancestral species, *Mycobacterium prototuberculosis*, before the emergence of the remaining MTC members.<sup>53, 72</sup> HGT has probably played an important role in the transfer of pathogenicity islands, from other environmental bacteria, that may have conferred a selective advantage in colonization of protozoa.<sup>72</sup> Rosas-Magallanes *et al* have shown that an operon essential for successful macrophage infection has probably been acquired through HGT by the MTC ancestor from a  $\gamma$ -proteobacterium donor.<sup>73</sup> Furthermore, Becq *et al* in a genome-wide comparative study of *M. tuberculosis* H37Rv, CDC1551 and *M. bovis* AF2122/97, detected 48 genomic islands probably acquired by HGT and, found that 8.2% of the genes present in these are necessary for *in vivo* virulence.<sup>74, 75</sup> In this view, it was proposed that current MTC members appear to no longer acquire new genomic regions by HGT, or at least do it at an extremely low rate, probably in part due to their restricted ecological niche.<sup>72, 76</sup> A recent publication, using high-throughput sequencing data from *M. tuberculosis* clinical isolates and comparison with other mycobacterial genomes suggests that recombination is still occurring in contemporary MTC members, contributing to genomic diversification.<sup>77</sup>

In the MTC organisms, the main mechanism of genomic diversification is probably through large genomic deletions as it has been shown that the MTC species have evolved through genomic downsizing.<sup>3, 78-81</sup> Some of these deletions are considered to be phylogenetically



informative, allowing the distinction of different species belonging to the MTC and differentiation of isolates from *M. tuberculosis sensu stricto* and its evolutionary history.<sup>3, 82</sup> The phylogeny obtained by Gagneux *et al* is also congruent with the SNP-based phylogeny and possibly reflects better the first human migrations.<sup>2, 78</sup> Based on phylogenetically informative deleted regions, Large Sequence Polymorphisms (LSP) or Regions of Difference (RD), these authors have defined six main lineages, two of which include the species traditionally known as *M. africanum* (Figure 1.8). Another study aimed at defining global phylogeography through 12 *loci* Mycobacterial Interspersed Repetitive Unit - Variable Number of Tandem Repeats (MIRU-VNTR) defines three major phylogenetic lineages within *M. tuberculosis sensu stricto*, congruent with Gagneux's LSP classification.<sup>83</sup> An integration of the different genetic marker based classifications is presented in Table 1.4.



**Figure 1.8** – RD-based phylogeny for different MTC lineages with greater resolution within *M. tuberculosis*. Branch rectangles indicate the clone-specific RDs. Also shown, is the geographical distribution of each lineage. Adapted from Gagneux *et al*.<sup>78</sup>

**Table 1.4** – Integration of different phylogenetic nomenclatures based on different markers and geographical association. Adapted from Gagneux & Small.<sup>84</sup>

Genetic Marker	Lineage 1	Lineage 2	Lineage 3	Lineage 4	Lineage 5	Lineage 6
<b>SNP</b> <i>Sreevatsan et al</i> <sup>52</sup>	PGG1	PGG1	PGG1	PGG2-3	PGG1	PGG1
<b>LSP</b> <i>Gagneux et al</i> <sup>78</sup>	Indo-Oceanic lineage	East Asian lineage	East African-Indian lineage	Euro-American lineage	West African lineage I	West African lineage II
<b>SNP</b> <i>Filliol et al</i> <sup>64</sup>	SCG 1	SCG 2	SCG 3a	SCG 3b-6b	Not done	Not done
<b>Spoligotyping</b> <i>Brudey et al</i> <sup>55</sup> <i>Demay et al</i> <sup>60</sup>	EAI	Beijing	CAS	Haarlem, LAM, T, X	AFRI2, AFRI3	AFRI1
<b>MIRU-VNTR</b> <i>Hill et al</i> <sup>83</sup>	Indo-Oceanic	East Asian and African Indian	East Asian and African Indian	Euro-American	West African I	West African II
<b>Geographical Association</b>	East Africa, Southeast Asia, South India	East Asia, Russia, South Africa	East Africa, North India	Americas, Europe, North Africa, Middle East	Ghana, Benin, Nigeria, Cameroon	Senegal, Guinea-Bissau, The Gambia

Besides the existence of phylogenetic informative deletions, some deletions have been found to be acquired independently in different lineages (homoplasies).<sup>66</sup> These deletions probably represent polymorphisms with phenotypic impact and might occur and be selected due to selective pressures favouring that specific impacted phenotype. Newton *et al*, *e.g.*, have identified an Asian lineage defined by a deletion later associated with immune subversion.<sup>85</sup>

Other factors such as polymorphisms in recombination, replication and repair genes (3R) may account for the species' diversification and adaptation. Mutations in these genes, that assure the genome's stability, might lead to a mutator phenotype and contribute to the development

of mutations in other genes at an increased frequency. It is possible that this phenotype might contribute to an improved adaptation to environmental changes, *e.g.* anti-TB therapy.<sup>86</sup>

Moreover, the location of different insertion sequences, such as IS6110, might also affect the expression and integrity of specific genes.<sup>87</sup> This insertion sequence has been widely used as a molecular marker in molecular epidemiology given its highly variable nature, in number and differential position in the genome. Soto *et al*, when analysing the insertion site of IS6110 of the MDR *M. bovis* B strain have verified that the insertion of an IS6110 in the promoter region of the *phoP* gene was responsible for the overexpression of this virulence associated gene.<sup>88</sup> In a similar way, Otal *et al* have also verified that in *M. bovis* isolates, the differential location of several IS6110 might constitute an adaptation factor to the host.<sup>89</sup> Safi *et al* further demonstrated that when *M. tuberculosis* grows inside human monocytes, the IS6110 effectively acts as a promoter of upstream genes.<sup>90</sup>

Other polymorphisms found throughout the genome of different strains might provide comparative information essential to understand and gain insights on the basis of human colonization, infectiousness and virulence.<sup>91</sup> Hirsh *et al* have observed that an association exists between the different *M. tuberculosis* strains and the human populations to which they are genetically related. Such association appear to result from a co-adaptation or interaction between the human and bacterial genomes.<sup>76</sup>

Genomic and proteomic variations between strains can induce a more virulent phenotype or elicit different immune responses by the host.<sup>92</sup> Reed *et al* verified that a phenol glycolipid, produced by a subset of *M. tuberculosis* isolates belonging to the Beijing/W family, inhibits the release of key pro-inflammatory cytokines by the cells of the host's innate immune response.<sup>93</sup> <sup>94</sup> Manca *et al* found on the other hand, that *M. tuberculosis* CDC1551 induced in vitro and in vivo a more vigorous immune response.<sup>95</sup> More recently, Nahid *et al* showed that Euro-American lineage, sub-lineage RD724, was associated with more severe disease at baseline, and together with the East-Asian lineage was associated with lower bacteriological conversion after 8 weeks of treatment.<sup>96</sup> It becomes clear that genetically different strains can evoke different immunopathological events.<sup>97</sup> Further data on the genomic diversity and association with disease presentation and/or virulence, including early reports on differential virulence between different geographically isolated strains, has been extensively reviewed by Coscolla & Gagneux.<sup>98</sup>

## 1.4 TUBERCULOSIS INFECTION

### 1.4.1 Symptomatology and Mode of Transmission

TB as a disease can show a symptomatology that can vary depending on the infected individual or the affected organ. From a clinical perspective, primary TB is usually asymptomatic, but can, however, evolve to a millary or post-primary TB if the immune response is not enough to control the initial infection.<sup>99, 100</sup>

The general symptoms of respiratory active TB generally include tiredness, fever, feeling of discomfort and anorexia. The weight loss occurs along with the progression of the disease, accompanied by sweating and fever, particularly during the night. Fever is usually low but chronic. Coughing is the most common respiratory symptom, being persistent (> 3 weeks) with mucoid or purulent sputum and possibly accompanied by hemoptysis (coughing sputum with blood). As mentioned, some degree of variability in the presentation of these symptoms may occur with individuals that show less symptoms.<sup>99, 100</sup>

Besides the respiratory system, TB can affect any organ and in such cases it is designed as extra-pulmonary or non-respiratory TB. The symptoms vary accordingly with the affected organ, *e.g.*, infection of the vertebral spine may lead to pain and vertebral spine deformations.<sup>99, 100</sup>

TB is in the great majority of cases transmitted by contagion from an infected individual, with active TB, that by coughing, sneezing or exhaling expel aerosols that contain bacilli able to infect another susceptible individual. This type of infection occurs by aerial route, allowing the bacilli to reach the lungs and start a new infection cycle. The infection might still take place vertically during the gestation period by hematogenic path through the umbilical artery, or by the amniotic fluid that is ingested or aspirated in the uterus during birth. This latter type of transmission results in a case congenitus TB that is distinct of acquired neonatal infection that occurs by contact with an individual with respiratory tuberculosis.<sup>101-103</sup>

### 1.4.2 Infection by *M. tuberculosis*

In the process of infection, *M. tuberculosis* generally gains access to the alveoli through aerosols generated by coughing or sneezing. Larger droplets (> 5  $\mu\text{m}$ ) do not reach the alveoli

as they are retained by the ciliated epithelia of the respiratory tract. Only the 1-3  $\mu\text{m}$  sized particles are infectious, can remain airborne for several hours, and are able to reach the alveolar space, each carrying 1-3 bacilli.<sup>104, 105</sup> It is thought that the first contact of the bacillus is with resident macrophages, but it is still thought possible that these bacteria are initially phagocytosed by type II pneumocytes.<sup>15</sup> Nevertheless, alveolar macrophages constitute the initial defence against *M. tuberculosis* infection; phagocytosis is mediated by contact of the macrophage mannose receptors and mannosyl groups present in the cell wall mannosylated LArM molecules or, through the complement receptors by opsonization of C3 and via CR3.<sup>106,</sup>

107

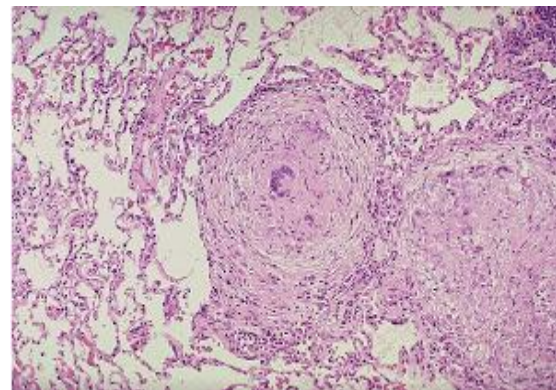
After phagocytosis, *M. tuberculosis* will initially reside in an endocytic vacuole, designated by phagosome. In normal circumstances, the fusion between phagosomes and lysosomes takes place, generating a hostile environment with an acidic pH, reactive oxygen and nitrogen intermediates (in particular, nitric oxide), lysosomal enzymes and toxic peptides. However, pathogenic mycobacteria inhibit the phagosome-lysosome fusion and still avoid phagosomal acidification.<sup>15, 106, 107</sup> The existing mannosylated lipoarabinomannan molecules on the mycobacterial cell wall appear to be important in the acidification blockade since it indirectly interferes, through the phosphatidylinositol 3-kinase, in the accumulation of syntaxin 6 that mediates the incorporation lysosomal hydrolases and vacuolar protonic ATPases in the phagosome.<sup>108, 109</sup> Trehalose 6,6'-dimycolate and the phenolic glycolipids are also essential in blocking the phagosomal acidification.<sup>110</sup>

Infected macrophages, through cytokine production, attract monocytes, lymphocytes and inactive neutrophils that help macrophages in the infection control. CD4<sup>+</sup> T lymphocytes recognize processed antigens processed in phagosomes and presented in the form of small peptides by MHC class II molecules in antigen presenting cells such as monocytes, macrophages or dendritic cells. This amplifies the immune response through the production of cytokines that attract more immune cells and, activate effector cells. It is the case for macrophages that once activated, will dispose of several cellular mechanisms to face off the infection such as the reactive oxygen and nitrogen intermediates.<sup>111</sup> CD8<sup>+</sup> T lymphocytes on the other hand, recognize peptidic fragments of cytosol-processed antigens exposed by MHC class I molecules, present in all nucleated cells, leading to the lysis or apoptosis of these infected cells.<sup>106</sup> Also important in the host's immune response are the dendritic cells that have the ability to phagocyte *M. tuberculosis* bacilli, and although they don't have a bactericidal capability towards this microorganism, they are able to maintain *M. tuberculosis* in a non-replicative state inside the phagosome. The dendritic cells are antigen presenting cells

that in the lymph node will induce the activation and differentiation of *naïve* T lymphocytes in effector cells. The dendritic cells also produce pro-inflammatory cytokines such as Tumor Necrosis Factor (TNF) and IL-6 that promote the synthesis of TNF and Interferon (IFN)- $\gamma$  by T lymphocytes, stimulating the macrophage-mediated antimicrobial activity.<sup>110</sup>

It was verified *in vitro* that three different outcomes might occur after phagocytosis: necrosis, in which cell death occurs with plasma membrane degradation; apoptosis, cell death in which the plasma membrane is preserved; or, survival of infected macrophages. Virulent *M. tuberculosis* strains subvert the regulation of prostaglandin E<sub>2</sub> (pro-apoptotic) and lipoxin A<sub>4</sub> (pro-necrotic) eicosanoids, promoting the necrotic pathway over the apoptotic pathway, with the latter being the most important not only in the reduction of bacterial viability but also, in the establishment of T cell-mediated cell immunity.<sup>112, 113</sup> Necrosis will on the other hand facilitate bacilli release and cellular reinfection.<sup>110</sup>

Focal granulomatous lesions are formed, composed by giant cells derived from macrophages and lymphocytes (Figure 1.9). The granuloma is initially composed by an amorphous mass of monocytes, neutrophils and macrophages. The macrophages later differentiate in nucleated giant cells (Langhans Cells), foamy macrophages and, epithelioid macrophages. The appearance of specific lymphocytes only occurs 2-3 weeks after infection. In this way, the propagation of the



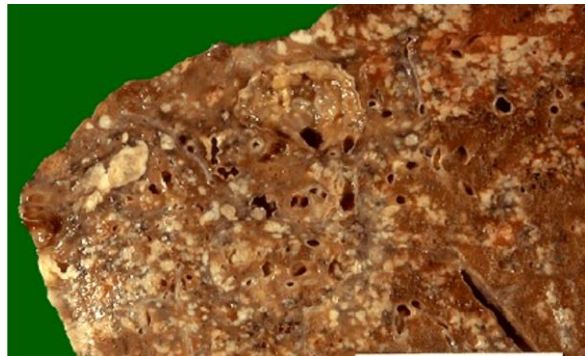
**Figure 1.9** – Microphotography of a granuloma and associated necrosis. Extracted from Luna.<sup>114</sup>

bacteria is generally contained and as cell immunity develops, bacilli-containing macrophages are killed, leading to the formation of the caseum in the centre of the granuloma, surrounded by a cellular layer composed by fibroblasts, lymphocytes, neutrophils and monocytes that is correlated with a bacilli-mediated local dysregulation of the host's lipidic metabolism.<sup>115</sup>

The granuloma has traditionally being viewed as a structure designed to protect the host and contain the bacterial replication. Nevertheless, the granuloma might end-up by protecting the bacilli on the inside from the immune system cells' and hamper the action of the antibacillary drugs.<sup>116, 117</sup> In fact, *M. tuberculosis* appears to have an active role in inducing the development of the granuloma by inducing the expression of the host's matrix metalloproteinases, important

in the degradation of the lung cell matrix and, simultaneously inhibit the expression of tissue metalloproteinase inhibitors.<sup>116</sup>

It is thought that *M. tuberculosis* is able to remain in a dormant state for decades on the inside of the granuloma, thus ensuring the persistence of the infection.<sup>118</sup> The foamy macrophages, called this way due to the accumulation of lipidic bodies, lose their bactericidal and phagocytic capability and may act as a niche for bacterial persistence.<sup>117</sup> In fact, it has recently been



**Figure 1.10** – TB affected lung showing necrosis and associated cavitation. Extracted from Luna.<sup>114</sup>

shown that *M. tuberculosis* induces the foamy phenotype by diverting the glycolytic pathway towards ketone body synthesis, leading to the accumulation of lipidic bodies.<sup>119</sup> The latency state is characterized by a reduced metabolic rate and is triggered by the expression of the DosR regulator, that controls a regulon of approximately 50 genes.<sup>120</sup> The expression of DosR and consequent activation of the regulon is the result of the response to three signals: hypoxia, nitric oxide and carbon monoxide.<sup>121-125</sup>

If the infected individual possesses an efficient cell immunity, the infection can be stopped at this stage, the granuloma heals resulting in small fibrous and calcified lesions.<sup>15</sup> In this granuloma late state, the caseum becomes hypoxic, and might induce a dormancy state or non-replicative persistence.<sup>126</sup> If however, an infected individual with a latent infection develops a weakened immune system due to HIV infection, immunosuppressive drugs, malnutrition, aging, etc., the inside of the granuloma is liquefied by an unknown process and becomes a rich medium for the growth of *M. tuberculosis*. The granuloma rupture contaminates the airways and the propagation of this liquid through the lungs leads to the development of cough, facilitating transmission, turning the individual into an infectious patient (Figure 1.10).<sup>15, 63</sup> It is also possible that bacterial propagation to other tissues may occur by hematogenic or lymphatic way and, cause extrapulmonary TB.<sup>15</sup>

### 1.4.3 HIV/TB Coinfection

The coexistence of HIV and *M. tuberculosis* in the same host represents at the present one of the more pressing dangers in public health. Each one of these pathogens alters the way in which the other interacts with the immune system potentiating its pathogenicity.

The WHO estimates that in 2010 there were 34 million HIV infected people, having occurred 2.7 million new cases worldwide in the same year. The incidence of the HIV infection has shown a global decreasing tendency since the late 90s but in some regions it appears to have again taken a growing tendency. This is the case of North Africa, Middle East, Eastern Europe and Central Asia.<sup>127</sup>

After the infection by *M. tuberculosis*, the progression rate to active disease is approximately 10% in immunocompetent individuals, half of which develop the disease in the two following years after infection. However, the risk of disease progression in immunosuppressed individuals by HIV infection is 5-15%/year.<sup>128, 129</sup> This substantially higher risk in coinfecting individuals results from different HIV pathogenicity inherent mechanisms not only due to the depletion of CD4+ T lymphocytes and consequent decrease of cellular responsiveness. Examples of these mechanisms consist in the reduction of the macrophage oxidative burst, overexpression of *M. tuberculosis* entry cell receptors or the diminishing apoptotic response by macrophages, essential for the elimination of intracellular bacilli.<sup>130, 131</sup>

At the pathological level, while in immunocompetent individuals TB is generally limited to the lungs, in HIV infected patients TB can present as a systemic disease affecting multiple organs. All the forms of extrapulmonary TB have already been described in HIV infected patients. Even at the pulmonary level the majority of the patients do not exhibit the typical granulomatous organization, but more diffuse lesions with granulocytic infiltrate. Cavitory lesions are only rarely found in patients with CD4+ T lymphocytic counts  $<200/\text{mm}^3$ .<sup>128, 129</sup>

On the other hand, *M. tuberculosis* equally potentiates the HIV pathogenicity inherent mechanisms and disease progression. It has been described that the *M. tuberculosis*-HIV coinfection has as a consequence an increase in the viral replication, which is reflected in a higher viral load in HIV infected individuals with active TB.<sup>132, 133</sup> This increase in viral replication might be due to the induction of TNF and IL-6, caused by the mycobacterial LArM, that through the NF- $\kappa$ B pathway leads to the transcriptional activation of the proviral long terminal repeats (LTR) promoter.<sup>129</sup>



*M. tuberculosis* also promotes the HIV infection through the overexpression of the CXCR4 and CCR5 coreceptors; induction of pro-inflammatory cytokine release, mainly the TNF; and, the decrease of the CCL5 expression.<sup>130</sup> Ku *et al* also demonstrated that TB affects the CD4<sup>+</sup> T lymphocyte count kinetics since in co-infected patients, the recovery of CD4<sup>+</sup> T lymphocyte to levels >250 cells/mm<sup>3</sup> is delayed.<sup>134</sup>

## 1.5 TUBERCULOSIS DIAGNOSIS

TB diagnostic is a process involving several stages and can be divided in two phases: the initial and definitive diagnostic. The initial diagnostic is done based on clinical data such as patient's symptoms and radiological data.

Definitive diagnostic implies a laboratory diagnosis performed by mycobacteriology specialized laboratories using the patient's clinical samples, *e.g.*, sputum or biopsy. The present criteria for laboratory confirmed TB cases imply a positive culture for MTC or, positive microscopy for acid-fast bacilli and detection of MTC specific DNA through a nucleic acid amplification test (NAAT).

### 1.5.1 Microscopy

Microscopy is based on the differential detection of acid-fast bacilli. This property is not exclusive of mycobacteria and it might be further observed in member species from the *Actinomyces*, *Nocardia*, *Corynebacterium*, *Rhodococcus* genera and, in oocysts from *Cryptosporidium* spp, *Isospora belli* and *Carcystis* spp.

The development of the acid-fastness staining method applied to TB diagnostics had its beginnings in the late 19th century with the works of Robert Koch, Georg Rindfleisch, Paul Ehrlich and, subsequent development by Franz Ziehl and Friedrich Neelsen for acid-fast bacilli (AFB) detection.<sup>135</sup> The Ziehl-Neelsen staining consists in a hot carbol-fuchsin stain, decolorizing with acid and counterstaining with methylene blue, malachite green or picric acid. Several variations of this Ziehl-Neelsen stain have come up through the years, usually differing on the decolorizing agent and solvent. The acids usually employed for the decolorizing purpose are the sulphuric acid at 25%, diluted in water, or chloridric acid at 3% in ethanol.<sup>99, 136</sup>

Three major modified versions of the Ziehl-Neelsen staining procedure exist and are the most widely used: the traditional Ziehl-Neelsen staining, the Kinyoun staining and, Truant's Auramine-Rhodamine staining. The Kinyoun staining is a modified version of the Ziehl-Neelsen staining, very similar to the original, except the fact that the carbol-fuchsin solution is used at a much higher concentration avoiding the use of heating in the primary staining procedure.<sup>99, 136</sup> However, in a study by Somoskövi *et al* comparing the three techniques has shown that the Kinyoun staining was the only that has produced false-negative results, while the Ziehl-Neelsen and the Auramine-Rhodamine stainings have shown comparable specificities and sensitivities.<sup>137</sup>

The Auramine-Rhodamine stain uses two fluorescent dyes (Auramine O and Rhodamine), although Auramine O can be used alone, by which a fluorescence microscopy is required. Slide examination time is reduced since it can be performed with lower magnifications (250X vs 1000X), and the same preparation can be confirmed and examined using the Ziehl-Neelsen stain. Ba *et al* has demonstrated that the mean time to declare a slide as negative using the Auramine-Rhodamine stain was 3 minutes and 44 seconds vs 7 minutes and 44 seconds using the Ziehl-Neelsen staining procedure.<sup>138</sup>

Microscopy has, nevertheless, a reduced sensitivity when compared with culture detection, only allowing the detection in samples with  $10^4$ - $10^5$  AFB/ml.<sup>139</sup> In the microscopy context, the concentration by centrifugation assumes special importance in the technique's sensitivity.<sup>140</sup>

## 1.5.2 Culture

Culture isolation of clinical isolates or strains of any MTC member is a time-consuming process that requires specially developed culture media. The TB bacillus was initially isolated in coagulated serum. Presently, diverse culture media are employed for this purpose, synthetic or complex and selective or non-selective.<sup>99, 136</sup>

Comparing with microscopy, culture isolation allows to posteriorly identify the isolate, eliminating the non-specificity problem of microscopy and, shows a higher sensitivity as it allows detection in samples with 10-100 AFB/ml.<sup>139</sup>

There are several formulations for the egg-based complex media, the most commonly used being the Löwenstein-Jensen (LJ), with several variations, *e.g.*, the Ogawa medium (no

asparagine) or Coletsos. This type of media are solidified by inspissation at 80-85°C and include, similarly to agarized media, malachite green, that confers some selectivity to the medium avoiding the growth of contaminating bacteria. The Petragami medium, mostly destined to the isolation of mycobacteria from samples with a higher contamination degree, has a higher malachite green concentration. The American Trudeau Society medium (ATS Medium) has, on the other hand, a decreased concentration of malachite green and is therefore mostly destined to the isolation of strains from usually sterile clinical samples, *e.g.*, cerebrospinal fluid.<sup>136</sup>

Posteriorly to LJ, several formulations of synthetic media have been developed, mostly by the works of Middlebrook, Dubos and Cohn.<sup>141</sup> The Middlebrook 7H9 (liquid) and 7H10 (solid, agarized) are the most diffused culture media, having the advantage, regarding the previous media, to allow the incorporation of antibacillary drugs for drug susceptibility testing. These media are usually supplemented with glycerol (2%, except for *M. bovis*), oleic acid, albumin, dextrose and catalase. There is also the 7H11 medium that differs from 7H10 by containing 0.1% casein hydrolysate, allowing an improved recovery of INH-resistant isolates. Both types of media can be supplemented with antimicrobial agents to ensure a higher selectivity, *e.g.*, the LJ Gruft contains nalidixic acid and penicillin.<sup>136</sup>

Growth on solid medium is however a time-consuming process due to the low replication rate of all members of the MTC. In the last decades, (semi-)automatized liquid culture systems have been developed, allowing a faster detection and diagnosis.

The two most widely used systems have been the BACTEC™ 460 (Becton Dickinson™) and the BACTEC™ MGIT™ (Mycobacteria Growth Indicator Tube) 960. The first is based on the radiometric detection of <sup>14</sup>CO<sub>2</sub> released due to the metabolism of palmitic acid radioactively labelled. The BACTEC™ 460 is however at a discontinuation stage but has been the gold standard for more than two decades.

The BACTEC™ MGIT™ 960 or the 320 version, more recent and with a lesser capacity, is now considered the gold standard for mycobacteria culture detection. This system is based on the detection of emitted fluorescence by a compound present in the culture tube that is O<sub>2</sub> sensitive. As the O<sub>2</sub> is consumed, the fluorescence increases indicating bacterial growth.

The mean time to detection in both systems is comparable (10-15 days) but is significantly less than growth detection on solid medium (20-26 days), particularly on AFB negative smears (16-18 vs 42 days).<sup>142-144</sup>

### 1.5.3 Drug Susceptibility Testing

Several methods have been traditionally employed in testing drug susceptibility to the different antibacterial drugs used in TB treatment, namely, the absolute concentration method, the resistance ratio method and the proportion method.<sup>145</sup> The proportion method has been adopted as the gold standard for TB drug susceptibility testing (DST). The rationale behind the proportion method is to assume that regarding resistance/susceptibility phenotype, natural variability exists in a *M. tuberculosis* population in its host but, if not more than a critical proportion (usually 1%, depends on the media and drug tested) of the population is able to grow in the presence of a drug, the calculated proportion is not of clinical significance and the strain is considered susceptible to the action of the tested drug.<sup>145</sup>

Standard DST by the proportion method was originally performed on egg-based media and, later, on agar-based media (preferred, *e.g.*, Middlebrook 7H10 or 7H11) through inoculation of several inocula dilutions in drug-containing and drug-free media and count of colony forming units (CFUs).<sup>145, 146</sup> The proportion of resistant CFUs is calculated by dividing the number of CFUs in drug containing medium by the number of CFUs in the drug free control, multiplied by 100. If the resistant CFUs account for more than the considered critical proportion of the total CFUs that develop on drug-free media, the strain is considered susceptible to that drug.<sup>145-147</sup> The concentration at which the drug is tested is called critical concentration, and is used as a breakpoint for classification of a strain as resistant or susceptible. This concentration is defined as the concentration that inhibits  $\geq 95\%$  of wild-type strains not previously exposed to the drug.<sup>148</sup>

The proportion method has also been adapted for commercial liquid-culture systems, such as the BACTEC 460 and BACTEC 960, in which the bacterial suspension is inoculated in a vial or tube with drug containing medium at the appropriate critical concentration. Furthermore a growth control is also established through the inoculation of a 1:100 dilution of the bacterial suspension. Briefly, if the bacterial growth rate in the drug medium is higher than the growth rate registered for the control vial or tube, the strain is considered resistant.<sup>146</sup> Moreover, the liquid proportion method is less likely to be as influenced by bacterial clumping as the proportion method in solid media.<sup>149</sup>

Susceptibility testing to PZA is performed differently than for other drugs as PZA is only active at an acidic pH and most reliable results are obtained using commercial systems with slightly acidic medium (pH=6).<sup>146</sup> The least reliable PZA drug susceptibility test is the one performed on acidic 7H10/11 supplemented with albumin, dextrose and catalase (no oleic acid as it is toxic at an acidic pH).<sup>150</sup>

A quantitative DST assay has been more recently developed through the use of BACTEC 960 equipped with specially developed software that monitors and automatically stores the growth units and plots the growth rates.<sup>151</sup> Comparison of growth rates in presence of different drug concentrations against a 1:100 diluted growth control allows classification as resistant, susceptible or intermediate resistant for each of the tested drug concentrations and, assess the strains' level of resistance (low-, intermediate- or high-level).<sup>151</sup>

#### 1.5.4 Nucleic Acid Amplification Tests

Due to the fact that the detection of the TB bacillus in clinical samples is a time consuming process and bacilloscopy is a method with a reduced specificity, nucleic acid amplification tests (NAAT) are equally employed in laboratory diagnosis. The NAAT are based on PCR amplification and detection of specific DNA or RNA from the MTC members.

Early NAAT tests included the Amplicor™ (Roche™) and the Enhanced Mycobacterium tuberculosis direct (E-MTD™) test (Gen-Probe™). The Amplicor test was based on the amplification and MTC-specific detection of the 16S rRNA gene, but has been discontinued. The E-MTD test is now on its second version, the amplified MTD test, and is based on the extraction, amplification and MTC-specific detection of rRNA by chemiluminescence. For this latter test, several studies report overall sensitivities of about 89-100%, although decreased susceptibilities are reported on negative AFB samples.<sup>152-162</sup>

Presently, the more diffused tests are based on specific amplification and reverse hybridization in strips with immobilized probes, *e.g.*, GenoType MTBSRplus (Hain Lifescience) and INNO-Lipa Rif. TB (Innogenetics™). Both tests allow detection from the clinical sample and screen for mutations associated to RIF and, for GenoType MTBDRplus, also with INH. Several published studies appear to demonstrate that the sensitivity of both methods is comparable (82-95%), although usually inferior in negative AFB samples or non-respiratory samples with decreased bacillary load.<sup>163-171</sup> Also commercialized are tests that allow the sole detection of MTC species

(GenoQuick MTB, Hain Lifescience) or specific non-tuberculous mycobacteria (GenoType Mycobacteria Direct, Hain Lifescience).

In recent years a new automatic system, GeneXpert™ MTB/RIF (Cepheid™), in which amplification and detection is performed in an almost fully automated way in a cartridge through a microfluidics system based on real-time PCR.<sup>172</sup> This system allows detection of MTC bacilli and RIF associated mutations in two hours, with a minimal hands-on time. Several studies have demonstrated specificities of approximately 99% and sensitivities above 90% on AFB positive samples, but decreased sensitivities in extrapulmonary or AFB-negative samples.<sup>173-188</sup>

In a recent study by Skenders *et al* it was demonstrated that in a total of 107 patients with positive bacilloscopy, the implementation of the INNO-Lipa Rif. TB test in the diagnosis routine has allowed to speed-up the empirical treatment but not the time or rate of culture conversion.<sup>189</sup>

### 1.5.5 Immunological Testing

Immunological testing presents an alternate/additional route to TB diagnostics, particularly in the case of latent tuberculosis infection (LTBI). The traditional method for LTBI diagnosis has been the tuberculin skin test (TST), in which an *M. tuberculosis* purified protein derivative (PPD) or tuberculin is usually injected intradermically. If the individual has come in contact with the tubercle bacillus an induration is expected to develop as a result of antigen recognition by the immune system. The early tuberculin or old tuberculin was initially developed by Robert Koch and consisted in a protein mixture extracted from boiled *M. tuberculosis* cultures. As this old tuberculin contained variable proportions of different proteins, which depended on the production method, a standardized tuberculin preparation was developed by Seibert *et al*, designated Purified Protein Derivative (PPD) and the international standard designated as PPD-S (standard) or PPD-M (mammal).<sup>190</sup> TST has however known limitations regarding cross-reactivity with other environmental mycobacteria and BCG vaccination.

In the last decade, the development of IFN- $\gamma$  release assays (IGRA) has resulted in two commercial tests for TB and LTBI diagnosis: QuantiFERON™-TB Gold In-tube (QFT-GIT) (Cellestis Ltd, Carnegie, Australia) and T-SPOT™.TB (T-SPOT) (Oxford Immunotec, Abingdon,

UK). QFT-GIT is a whole-blood test based on Enzyme-Linked Immunosorbent Assay (ELISA) to quantitatively detect IFN- $\gamma$  produced by lymphocytes upon overnight exposure to three TB antigens: ESAT-6, CFP-10 and TB7.7. In T-SPOT, peripheral blood mononuclear cells (PBMCs) are separated, counted and exposed to two TB antigens: ESAT-6 and CFP-10. The production of IFN- $\gamma$  is assessed by culture supernatant ELISA in QFT-GIT and by enzyme-linked immunospot (ELISpot) in T-SPOT. In both IGRA tests no cross-reactivity with BCG vaccination is expected since all antigens that are used in both tests belong to the RD1 (ESAT-6 and CFP-10) or RD11 (TB7.7) regions that are absent from *M. bovis* BCG strain.

Recent systematic reviews and meta-analysis of published data show that overall pooled sensitivity may range between 61-69% for QFT-GIT and 65-66% for T-SPOT although stratification by high/low-burden setting may yield slightly different results: in high-burden settings, 61-65% for QFT-GIT and, 65-72% for T-SPOT; in low-burden settings, 59-67% for QFT-GIT and, 69-94% for T-SPOT.<sup>191-194</sup> Overall specificity ranged between 72-76% and 63-70% for QFT-GIT and T-SPOT, respectively. In low-burden settings, specificity ranged between 89-94% for QFT-GIT and was 64% for T-SPOT, while in high-burden settings it ranged between 50-62% and 52-73% for QFT-GIT and T-SPOT, respectively.<sup>191-194</sup> Except for Chen *et al* that found statistically significant higher specificity value for QFT-GIT in low-burden settings, none of the differences reported in sensitivity or specificity is statistically significant.<sup>191-194</sup>

Although T-SPOT is generally regarded as a more sensitive technique, the exclusion of failed tests due to insufficient number of PBMCs may contribute to the overestimation of the test's sensitivity in HIV-infected patients.<sup>191</sup>

Another important aspect is the proportion of indeterminate results and several factors can affect this parameter: younger age; malaria, HIV or helminth infection; iron deficiency; iron deficiency anaemia or HLA allele.<sup>195, 196</sup> In the same recent systematic reviews and meta-analysis, overall pooled proportion of indeterminate results was 8.2-10.0% and 5.9-13.2% for QFT-GIT and T-SPOT, respectively. Although both tests had generally higher proportion of indeterminate results in high-burden settings: 4-15% (QFT-GIT) and 2-14% (T-SPOT) vs 4-8.4% (QFT-GIT) and 0-5% (T-SPOT), for high- and low-burden settings, respectively, no significant differences were found.<sup>191-194</sup> Santin *et al* found a statistically significant higher proportion of indeterminate cases using QFT-GIT among patients with active TB (culture confirmed or symptomatic), 15.3%, in comparison with patients screened for LTBI, 3.9%.<sup>191</sup> A higher, but not statistically significant, difference in the proportion of indeterminate results was also found for

QFT-GIT (8.1%) in comparison with T-SPOT (4.0%) when using a patient stratification threshold of 200 CD4+ cells/ml.<sup>191</sup>

Most studies compare the performance of IGRA tests in patients with active TB and the data regarding the performance of these tests in diagnosing and screening LTBI is limited due to the lack of a gold-standard. However, two studies by Aichelburg *et al* and Santin *et al* showed that QFT-GIT had a 100% negative predictive value for development of active TB.<sup>197, 198</sup>

## 1.6 TUBERCULOSIS TREATMENT

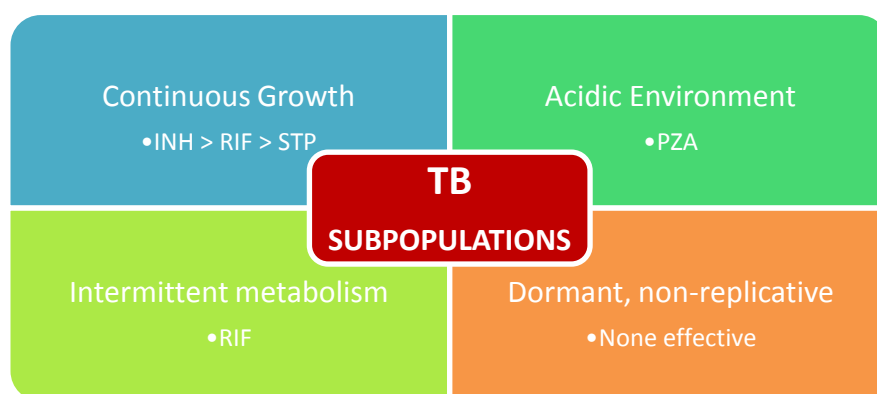
### 1.6.1 Tuberculosis Treatment Regimens

The antibacillary drugs available to fight TB are divided into first-line drugs and second-line drugs. The former present a higher efficacy, less side effects and are comprised by INH, RIF, ethambutol (EMB), STP and PZA. Second-line drugs are not as efficacious as first-line drugs, present a higher degree of side effects and include several drugs such as: KAN, AMK, CAP, FQs, ethionamide (ETH), para-amino salicylic acid (PAS), cycloserine (CS), clofazimine (CFZ) and prothionamide (PTH).

For TB drug therapy design it is necessary to acknowledge that different bacilli subpopulations exist in the host, concerning the bacterial metabolic state. According to the Mitchison's hypothesis, there are four different populations of bacilli with distinct metabolic states: the first, exhibit continuous growth and are actively dividing, comprising the large majority of the bacilli within the host with active TB; the second, is comprised by bacilli in an acidic environment, within macrophages or the caseum, in a state of semi-dormancy; the third subpopulation is composed by bacilli located extracellularly in solid caseous lesions, that exhibit intermittent growth; the fourth subpopulation is comprised by bacilli in a complete dormant state (persisters) that is, at the present, beyond the reach of any drug.<sup>199, 200</sup> To tackle these populations some of the available antibacillary drugs are more appropriate than others (Figure 1.11).<sup>199, 200</sup>

TB chemotherapeutic regimens can be divided in two stages or phases: the initial phase, that includes a higher number of drugs to achieve a rapid killing effect and sputum conversion, with the main purpose of reducing the patient's infectiousness; and, a longer continuation phase, with less drugs, that aim at a better sterilization effect and prevention of relapses.<sup>200</sup>





**Figure 1.11** - Schematic representation of the four different bacilli subpopulations that exist in a host and the first-line drugs that better target these, according with the Mitchison's hypothesis. Adapted from Khadivaran & Sharma, 2008.<sup>201</sup>

The cornerstone of TB treatment is the standard six-month regimen constituted by a two-month initial phase of INH+RIF+PZA+EMB and a four-month continuation phase of INH+RIF (former Category I). This regimen is recommended for every new patient with no MDR-TB suspicious, although EMB should be maintained throughout the continuation phase in settings with high primary drug resistance rates (*i.e.*, drug resistance not resulting from a previous treatment).<sup>202, 203</sup>

Another first-line treatment regimen, formerly Category II, was recommended in which STP or EMB were added alone, and both treatment phases extended by one additional month for relapses or re-treatments due to patient default. In the most recent recommendations, category II regimen with the addition of STP alone is still recommended, albeit with a different designation, for previously treated patients, including relapses and defaults, where the likelihood of MDR-TB is low or medium. If DST data becomes available this regimen should be adapted or modified; if not, it is recommended by the WHO to maintain the regimen for the full course of the treatment. Whenever the likelihood of MDR is high on a previously treated patient, it is recommended to go for an empirical MDR-TB treatment regimen.<sup>202, 203</sup>

The previously standard regimens recommended by the WHO for MDR-TB treatment of patients with suspicious MDR-TB, but with no DST data available or DST data for first-line drugs only are schematized in Table 1.5.<sup>202</sup>

**Table 1.5** – MDR-TB treatment regimens recommended by the WHO. Adapted from WHO guidelines.<sup>203</sup>

First-line DST	Initial Phase		Continuation Phase	
	Drugs	Duration (months)	Drugs	Duration (months)
Not available	KAN+ETH+FQ+PZA+/-EMB	>=6	ETH+FQ+PZA+/-EMB	12-18
INH+RIF	STP+ETH+FQ+PZA+/-EMB	>=6	ETH+FQ+PZA+/-EMB	12-18
All first-line drugs	1 Injectable Drug+ 1FQ + 2 of: PAS, ETH, CS	>=6	All except the injectable drug	18
Second-line drug DST available	Tailored regimen according with DST pattern			

According with the most recent WHO recommendations, each country should choose the most adequate MDR standard treatment regimen, based on surveillance data and drug resistance surveys.<sup>203</sup>

**Table 1.6** – Hierarchy of five drug groups that should be chosen for tailored MDR-TB treatment regimens. Extracted from WHO guidelines.<sup>203</sup>

Drug Groups				
1	2	3	4	5
PZA	KAN	Levofloxacin	PAS	CFZ
EMB	AMK	Moxifloxacin	CS	Linezolid
Rifambutin	CAP	Ofloxacin	Terizidone	Amoxicilin/Clavulanate
	STP		ETH	Thioacetazone
			PTH	Imipenem/Cilastatin
				High-dose INH
				Clarithromycin

For tailored regimens the WHO has defined five groups of drugs, that should be added to a regimen in a hierarchical order (Table 1.6).<sup>203</sup> At least four effective drugs should be added to a regimen following the group order. Only one drug can be chosen from groups 2 and 3, while as many drugs as possible, or necessary, can be chosen from the remaining groups. The remaining general principles for MDR-TB treatment design can be found further detailed in the most recent WHO guidelines.<sup>203</sup>

## 1.6.2 Antibacillary drugs

### INH

INH or isonicotinic acid hydrazide is a synthetic prodrug that requires activation by the bacterial catalase peroxidase, encoded by the *katG* gene.<sup>204</sup> Upon activation an isonicotinic acyl radical is produced that binds NAD, forming an INH-NAD adduct that targets the InhA enoyl-ACP reductase, essential for mycolic acid elongation by the type II fatty acid synthase system (FASII).<sup>205-207</sup> The inhibition of mycolic acid biosynthesis leads to cell lysis and death. INH enters the cell by passive diffusion and its activity is restricted to dividing bacteria under aerobic conditions.<sup>208</sup>

INH great efficacy is in part due to its low minimum inhibitory concentration (MIC): 0.02 mg/L for *M. tuberculosis* H37Rv and 0.02-0.05 mg/L in susceptible clinical isolates.<sup>209</sup> INH has a bactericidal activity against rapidly growing mycobacteria and bacteriostatic against slow-growers, although bactericidal activity is observed against *M. tuberculosis*, *M. bovis* and *M. kansasii*.

INH resistance usually develops as a result of *katG* mutations that decrease the ability of the catalase-peroxidase to convert INH to its active form. Depending on the mutations found, *katG* mutations may result in low to high-level resistance.<sup>210</sup> Resistance level is negatively correlated with the remaining KatG INH oxidase activity.<sup>210</sup> The most common mutation found in *katG* is a serine to threonine substitution at codon 315 (S315T), found in up to 93% of INH resistant isolates, associated with an INH high-level resistance.<sup>204, 211-234</sup> S315T has been shown to decrease KatG catalytic activity and binding affinity towards INH.<sup>235-237</sup>

Another important mechanism of INH resistance, usually second to *katG* mutations, consists in the acquisition of hypermorphic mutations in the promoter region of *mabA(fabG1)-inhA* operon in up to 32% of INH resistant isolates.<sup>215-221, 223, 224, 226-232, 234, 238</sup> Although such mutations usually lead to INH low-level resistance, an unusual high-prevalence of *inhA* promoter mutations have been detected in Lisbon, Portugal, and in *M. africanum* West-Africa 1 lineage.<sup>24, 223, 225</sup>

Furthermore, NAD<sup>+</sup>/NADH altered ratios, mediated by *ndh* mutations, can also result in INH and ETH resistance.<sup>207</sup> Mutations found in other genes, namely *kasA* and *ahpC*, have an unknown or questionable role in INH resistance.<sup>223, 227, 230, 232, 234</sup> Some studies have however

reported up to 35% of INH resistant clinical isolates without mutations in any of the above genes, suggesting alternate mechanisms for INH resistance, such as drug efflux.<sup>227, 239-241</sup>

### RIF

RIF or rifampin is a semi-synthetic antibacillary drug derived from rifamycin introduced in TB chemotherapy in 1967.<sup>242</sup> RIF binds to the  $\beta$ -subunit of the DNA-dependent RNA polymerase, encoded by the *rpoB* gene.<sup>243</sup> Binding to the RNA polymerase  $\beta$ -subunit is thought to physically block transcription of growing RNA chains when these become 2-3 nucleotides in length.<sup>244, 245</sup> RIF has a bactericidal activity against most Gram-positive, some Gram-negative and mycobacteria.<sup>246, 247</sup> Active against metabolically active bacteria, RIF also possess some degree of sterilizing activity as it is active against latent bacilli with spurts of activity.<sup>242, 248</sup>

RIF MIC ranges between 0.2-0.4 mg/L for susceptible clinical isolates (0.4 mg/L for *M. tuberculosis* H37Rv).<sup>209</sup>

Acquisition of RIF resistance is usually the result of aminoacid substitutions in a 81-bp region of the *rpoB* gene named RIF resistance determining region (RRDR) or cluster I.<sup>249-251</sup> Besides aminoacid substitutions, deletions or insertions in *rpoB* have been reported in some studies.<sup>252, 253</sup> The most common substitutions occur in codons 531 (prevalence of 31.0-76.9% of the RIF-resistant isolates), 526 (7.7-43.0%) and 516 (3.4-28.6%).<sup>214, 215, 223, 230, 231, 245, 249, 250, 252-261</sup> Mutations in these three codons are thought to have the least impact on fitness, particularly S531L mutations.<sup>262, 263</sup>

RIF resistance level depends on the substituted residue, or combination of mutations, and the type of substitution, *e.g.*, S531L, H526D or D516V result in high-level resistance whereas D516Y results in low-level resistance.<sup>264-266</sup>

Up to 11% of RIF resistant isolates do not show any RRDR mutations, with some isolates displaying N-terminal mutations that may affect protein-drug interaction or, mutations in *rpoB* cluster II (codons 571-572).<sup>249, 261</sup>

A different mechanism involving inactivation of RIF by ribosylation in mycobacteria has been identified as partially responsible for the low susceptibility of some species to RIF, but no mechanism of this type has been described for *M. tuberculosis*.<sup>267, 268</sup>

## EMB

EMB is an antimycobacterial drug synthesized from ethylenediamine and used in TB treatment since 1968.<sup>242</sup> EMB targets the cell wall biosynthesis, more specifically inhibiting the arabinosylation of cell wall arabinogalactan (AG) and LAraM.<sup>269</sup> EMB targets the arabinosyltransferases encoded by the *embCAB* operon, but inhibiting the activity of EmbB with a greater affinity than EmbC. Since *embAB* gene products are responsible for the arabinosylation of AG whereas arabinosylation of LAraM is catalyzed by the *embC* gene product, EMB leads to a more rapid inhibition of AG biosynthesis than LAraM biosynthesis.<sup>270-273</sup> The EMB mediated AG biosynthesis inhibition leads to further mycolic acid accumulation due to the depletion of mycolate attachment sites.<sup>270</sup>

EMB enters the cell in a passive manner and, has a bacteriostatic activity against metabolically active bacilli rather than bactericidal.<sup>274-276</sup> EMB MICs range between 0.5-2 mg/L for susceptible isolates (0.5 mg/L for *M. tuberculosis* H37Rv).<sup>209</sup>

EMB resistance has been traditionally associated with *embB* mutations, of special incidence on a EmbB 142 aa extracytoplasmatic loop.<sup>277-280</sup> The most common mutations occur in codon 306 in up to 68% of EMB resistant isolates and usually involve the substitution of a methionine for a valine, leucine or isoleucine.<sup>219, 281-290</sup> Strains bearing *embB306* mutations have been associated with a higher level of EMB resistance than other *embB* mutations.<sup>286, 290, 291</sup> On the other hand, M306V/L mutations appear to confer a higher level of EMB resistance than M306I.<sup>219, 278, 285</sup>

In more recent years the utility of *embB306* mutations as molecular determinants of EMB resistance has been questioned as several authors reported EMB susceptible isolates bearing *embB306* mutations resistance whereas others only detected *embB306* mutations among EMB resistant isolates.<sup>219, 283, 284, 287, 292-294</sup> Allelic exchange experiments have shown that *embB306* mutations only moderately increase EMB MIC to levels below the ones observed in clinical isolates. Moreover, *embB306* mutations appear to be required for high-level resistance, highlighting that this level of resistance is the result of multigenic mutational events.<sup>295-297</sup> Other genes such as the *iniA* gene, essential to EMB efflux, may be involved in EMB resistance although the paucity of data in this regard prevents such associations.<sup>278, 298, 299</sup>

## STP

STP was the first antibiotic used in TB treatment, in 1944, and is an aminoglycoside isolated from *Streptomyces griseus*.<sup>242, 300, 301</sup> STP interferes with protein synthesis by inhibiting genetic translation with a more pronounced effect on elongation rather than initiation.<sup>211</sup> Crystallography of *Thermus thermophilus* 30S ribosomal subunit complexed with STP, shows that STP tightly binds 16S rRNA (*rrs* gene) through both salt bridges and hydrogen bonds to four different parts of the molecule and across ribosomal protein S12 (*rpsL* gene)<sup>302</sup> The binding of STP appears to stabilize the ribosomal ambiguity state (ram state) and have a negative effect on the ribosomal proof-reading activity.<sup>302, 303</sup> STP-induced mistranslation leads to an increased proteolysis rate and abnormal misread proteins appear to create protein channels causing loss of permeability control, accelerating STP irreversible uptake and block of translation initiation.<sup>304-307</sup> The stabilization of the peptidyl-tRNA in the A-site has also been demonstrated to reduce the translocation rate.<sup>308</sup>

The initial use of STP in monotherapeutic regimens has lead to the emergence of high number of resistant cases, and with subsequent development of more efficacious antibacillary drugs, STP gradually lost its role as a first-line drug, mainly in developed countries.<sup>309</sup>

STP MICs range between 1.0-2.0 mg/L (1.0 mg/L for *M. tuberculosis* H37Rv) and has a moderate bactericidal activity against susceptible isolates.<sup>209, 301</sup> On comparison with the two other aminoglycosides used in TB treatment, KAN and AMK, STP is the least toxic.<sup>310</sup>

Resistance towards STP is usually mediated by mutations in the *rpsL* gene, generally a substitution of a lysine by an arginine at codon 43, although mutations on codon 88 are often reported and associated with STP resistance.<sup>311</sup> Mutations in *rpsL* gene are associated with a high-level resistance, particularly K43R mutations.<sup>312-315</sup> Another mechanism of STP resistance is *rrs* gene mutations that generally occur in the 530 and 910 loops, which interact with STP and are in close proximity due to the 16S rRNA secondary structure.<sup>311</sup> Mutations in the *rrs* gene usually yield a resistance level below the resistance level of isolates bearing *rpsL* mutations, but high enough to be considered an intermediate resistance level, although MICs can vary significantly depending on the mutation.<sup>313, 314</sup>

The prevalence of each of these mutations displays a wide variability, depending on strain lineage or geography. Mutations in *rpsL* gene are usually more prevalent (up to 89%) than *rrs* mutations (0 – 28.6%).<sup>220, 287, 312, 315-324</sup> Nevertheless, in a study by Cuevas-Cordoba *et al*, *rrs*

mutations were found to be more prevalent than *rpsL* mutations, 28.6% vs 19.8%, respectively.<sup>317</sup>

The loss of the *gidB*-encoded rRNA methyltransferase function, found to be responsible for the methylation of G527 of the 16S rRNA, leads to a reduced affinity of STP towards the ribosome and STP low-level resistance.<sup>325, 326</sup> *GidB* mutations have been detected in clinical isolates, although the precise quantitative role in STP resistance is still unclear as some of the mutations detected in STP-resistant isolates are also present in susceptible ones.<sup>214, 327</sup> Other polymorphisms detected in *gidB* have been associated with strain lineage rather than STP resistance.<sup>327, 328</sup>

## PZA

PZA is a nicotinamide synthetic prodrug that has been introduced in TB treatment in 1970 and is important in the short-course TB treatment.<sup>242</sup> PZA is thought to enter the cell by passive diffusion where it is converted by the bacterial pyrazinamidase (PZase)/nicotinamidase into pyrazinoic acid (POA), that accumulates in the cell due to a deficient efflux system, only leaving the cell by passive diffusion in an acidic pH environment. Extracellular protonation of POA allows reentrance by passive diffusion where deprotonation leads to cytoplasm acidification.<sup>150, 329, 330</sup> The acidification of the cytoplasm may contribute to the depletion of the membrane energy potential.<sup>331</sup>

For many years the identification of a precise target of PZA was unsuccessful as attempts to isolate POA resistant mutants have failed.<sup>332, 333</sup> More recently, Shi *et al* found that PZA inhibits trans-translation through the targeting of the *rpsA*-encoded ribosomal protein S1 by POA.<sup>334</sup>

PZA has an excellent sterilizing activity as it is bactericidal against semi-dormant bacilli in an acidic pH. The introduction of PZA in conjunction with RIF has showed synergism and allowed the shortening of treatment regimens containing both drugs from 12 to 6 months.<sup>150, 335</sup>

The main mechanism behind PZA resistance is the acquisition of mutations in the *pncA* gene.<sup>336</sup> Mutations in the *pncA* gene have been identified in numerous studies in about 72.0-99.9% of the PZA resistant isolates studied.<sup>214, 219, 332, 337-354</sup> Most of the mutations identified are predicted to affect the enzymatic activity of PncA due to the alteration of the active site or destabilization of the protein structure leading to the loss of the PZase activity.<sup>353</sup> As the *pncA* gene is not essential to bacterial viability, the gene is under a less stringent mutational constraint leading to a high mutational diversity and independent arise of PZA resistance at a

lower rate ( $10^{-5}$ ).<sup>340, 342, 353, 355</sup> The resistance level to PZA depends on the mutations, although mutational hotspots have been identified, with the higher proportion in the loop between the  $\beta 2$  and  $\beta 3$  strands that comprise the metal coordination site of the enzyme's catalytic centre.<sup>332, 346, 356</sup> In fact, docking simulations show that some point mutations contribute to a higher resistance level than others.<sup>357</sup>

PZA resistant isolates with a wild-type *pncA* gene fall in two groups: PZase-negative and PZAase-positive isolates.<sup>338, 339, 347</sup> While PZA resistance in PZase-positive isolates may be explained by mutations at other *loci*, namely the target of POA, resistance in PZase-negative isolates with a wild-type *pncA* gene is probably due to a differential regulation of *pncA* gene expression.

Mutations in RpsA may also lead to PZA resistance through the inhibition of POA-RpsA binding.<sup>334</sup> Nevertheless, Alexander *et al* suggested that *rpsA* mutations are not robust markers for PZA resistance.<sup>337</sup>

### *Injectable Second-Line Drugs*

The injectable drugs of second-line drug use in TB treatment are KAN, AMK and CAP. Although KAN and AMK are, such as STP, aminoglycosides and CAP is a macrocyclic peptide from the tuberactinomycin family, these drugs share the same basic action mechanism and incomplete cross-resistance between the three has been well documented.<sup>358, 359</sup> KAN, AMK and CAP have shown *in vitro* bactericidal activities. Furthermore, CAP has a bactericidal effect against non-replicating *M. tuberculosis*.<sup>360</sup>

These three drugs act through the inhibition of the genetic translation due to ribosomal binding. Nevertheless, the exact mechanism through which these drugs inhibit the ribosomal activity during translation is not fully understood or studied to the extent that has been with STP. AMK and KAN have been shown to bind to the A-site of the ribosome and inhibit translation.<sup>361</sup> CAP mode of action has been initially deduced from early studies of ribosomal translation inhibition with its analogue viomycin (VIO) and, more recently confirmed by the crystal structure of CAP and VIO in complex with the 70S ribosomes.<sup>362-364</sup> These studies have shown that both VIO and CAP affect the dissociation of the 70S ribosome of *M. smegmatis* by stabilization of the 70S couples and, inhibit the translocation by arresting the peptidyl-tRNA in the ribosomal A-site.<sup>362-364</sup> CAP binds to the interface between helix 44 and helix 69 of the small (16S) and large (23S) ribosomal subunits, which is dependent on the methylation, by the



TlyA 2'-O-methyltransferase, of C1409 in helix 44 and C1920 in helix 69 of the 16S and 23S rRNA, respectively.<sup>363, 365</sup>

Mutations in the 16S rRNA-encoding *rrs* gene can mediate cross-resistance between CAP, KAN and AMK. The most common mutation found in the *rrs* gene is the A1401G mutation, present in about 49.3-88.6% of the resistant isolates, and associated with resistance to KAN and AMK, and CAP low-level resistance.<sup>215, 219, 234, 359, 366-370</sup> The C1402T mutation does, on the other hand, mediate resistance to KAN, CAP, VIO but not AMK whereas the G1484T mutation mediates resistance to all four drugs (KAN, AMK, CAP and VIO).<sup>359</sup>

KAN resistance has also been linked with *eis* overexpression, a gene encoding an acetyl transferase that can multi acetylate several aminoglycosides, rendering the drugs ineffective.<sup>371</sup> Since Eis acetylates KAN more efficiently than AMK, *eis* overexpression has been associated with KAN low-level resistance but not AMK resistance.<sup>372</sup> In this regard, several *eis* promoter mutations have been identified in clinical isolates.<sup>373, 374</sup>

CAP resistance can also be mediated by *tlyA* mutations.<sup>373, 375</sup> Maus *et al* have shown that loss of TlyA 2'-o-methyltransferase function causes CAP resistance and, Manshupanee *et al* have shown that TlyA N-terminal aminoacid substitutions reduce its ability to methylate C1409 and C1920 in the 16S and 23S rRNA and that subtle changes in the level of rRNA methylation lead to significant differences in the CAP sub-inhibitory concentrations.<sup>375, 376</sup> Nevertheless *tlyA* mutations in CAP resistant isolates are only seldom observed in CAP resistant isolates as it is more likely that CAP resistance develops as a result of KAN/AMK cross-resistance.<sup>368, 373</sup> Another possibility is that CAP resistance may be mediated by 23S rRNA mutations.<sup>365</sup>

## FQs

FQs are fluorinated quinolones at the central ring system. These are broad-spectrum antibacterial drugs that target the bacterial DNA gyrase and topoisomerase IV, therefore inhibiting DNA replication.<sup>377</sup> *M. tuberculosis* lack the topoisomerase IV *parE* and *parC* subunit homologues, limiting the inhibitory activity of FQs to the DNA gyrase through interaction with both GyrA and GyrB subunits.<sup>62, 378</sup> Quinolones bind to the DNA Gyrase-DNA binary complex producing a ternary complex that halts DNA replication leading to cell death.<sup>379</sup> The binding site of quinolones - the Quinolone Binding Pocket (QBP), constituted by protein and DNA, is located in the enzyme's catalytic core where the drug interacts with both the GyrA and GyrB subunits and is intercalated between the dinucleotide step.<sup>379</sup>

FQ resistance has been reported to be increasing in several settings and several studies have described an association between previous exposure to FQ prior to TB diagnosis and an increased risk of having FQ-resistant TB.<sup>380, 381</sup> Devasia *et al* also associated a previous FQ treatment for more than 10 days with FQ resistance.<sup>382</sup> This data seem to correlate with the fact that quinolones induce the bacterial SOS repair system, which is error-prone, and can induce the development of FQ resistance in *M. smegmatis* and *M. tuberculosis* with involvement of the SOS system.<sup>383</sup> Gatifloxacin (GTX) and moxifloxacin (MOX) appear to induce FQ resistant mutants at a lower rate than ciprofloxacin (CIP) and levofloxacin (LVF).<sup>383</sup> Furthermore GTX and MOX are more effective (lower MICs) than CIP and ofloxacin (OFX) and can be used to treat FQ low-level resistant isolates.<sup>384</sup> Von Groll *et al* has also observed that almost complete cross-resistance between OFX, MOX and GTX existed.<sup>385</sup>

The molecular basis of FQ resistance has been associated with *gyrA* and *gyrB* mutations in particular hotspots denominated Quinolone Resistance Determining Regions (QRDR).<sup>386</sup> *GyrA* QRDR is located between codons 74-95, whereas *GyrB* QRDR has been proposed to be comprehended between codons 500-540.<sup>271, 387</sup> Both QRDRs are located at the QBP according to the DNA gyrase crystal structure.<sup>379, 387</sup> The most common mutations associated with FQ resistance occur in *gyrA* in codons 94 and 90, in 46.2-71.9% and 4.0-38.5%, respectively.<sup>219, 367, 380, 384, 385, 388-391</sup> Mutations occurring in the *gyrB* gene have also been described, although with a lesser frequency and some with questionable role in FQ resistance.<sup>367, 378, 392</sup> Double mutations have also been described to act synergistically to confer FQ-resistance or, as associated with a reduced FQ susceptibility.<sup>378, 390, 391</sup>

Data from structural analysis show that *gyrA* and *gyrB* mutations can cause FQ resistance through the modification of the QBP geometry or lead to the modification of the DNA structure in the QBP when mutations in aminoacid residues that interact with the DNA occur.<sup>379</sup>

Regarding resistance levels, the wide range of MICs observed for a given mutation, different panels of tested FQs and, the diversity of methodologies employed in MIC determination hamper direct comparison.<sup>384, 389-391</sup> For instance, in a recent study, Cui *et al* have not found any statistical difference between MICs of strains with mutations on different *gyrA* codons or in *gyrB*.<sup>389</sup> Nevertheless several studies convey the notion that *gyrA* D94G/A/N mutations exhibit a higher resistance level than A90V and S91P, by this order.<sup>384, 389-391</sup> Differences in MIC-mutation correlations between these studies may also be related with the observation that FQ MIC can exhibit some degree of variability according with the strain's genetic background.<sup>378</sup>

Other mechanisms of FQ resistance might be involved as data from Singh *et al* showed that FQ efflux may play a role in FQ resistance.<sup>393</sup>

## 1.7 TB MOLECULAR EPIDEMIOLOGY

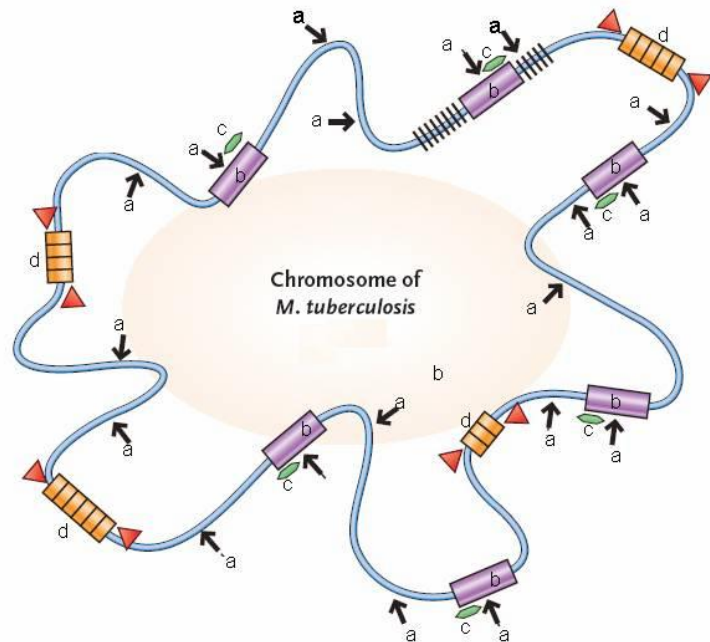
Molecular epidemiology can be defined as the study of the distribution and determinants of diseases that occur among human populations using molecular biology techniques.<sup>394</sup> The application of molecular methods in the genotyping of *M. tuberculosis* isolates has allowed obtaining more information on its propagation in miniepidemics or outbreaks.<sup>394</sup> Before molecular methods became available, strain discrimination relied on bacteriological data with reduced discriminatory power. The implementation of molecular data in the study of TB transmission, during the 80's, has allowed novel insights in TB epidemiology.<sup>395</sup>

The main principle behind TB molecular epidemiology relies in the assumption that high genotype variability exists among epidemiologically non-related *M. tuberculosis* isolates, while patients infected from a common source, or belonging to the same transmission chain, will have isolates with identical genotypes. Furthermore, isolates with identical or similar genotypes, *i.e.* clustered isolates, probably reflect cases that have arise due to recent transmission, whereas isolates with different genotypes reflect reactivation cases, of an infection acquired in the past, or even cases associated with migratory events.<sup>59</sup> Although the treatment of reactive cases or recent transmission cases is similar, recent transmission may be indicative of an outbreak or series of transmissions that require control measures in order to be contained.<sup>395</sup>

Genotypic data should, nevertheless, be interpreted at the light of the traditional epidemiologic information that is available and should consider the evaluation of a large percentage of the population's cases during a considerable time period.<sup>59</sup> This way, molecular epidemiology might provide additional data concerning TB transmission dynamics, distinguish exogenous reinfection from endogenous reactivation, determine the source of laboratory contaminations, identify risk factors for transmission in the community, and follow the geographical distribution and propagation of clones of public health concern.<sup>59</sup> Different reports have highlighted the importance of genotyping in improving traditional contact tracing in complex transmission networks where TB can be transmitted by brief contact and, in the identification of environments with increased risk for TB transmission.<sup>396-399</sup> Moreover,

molecular epidemiology is also invaluable in tracking the international dissemination and evolutionary changes in *M. tuberculosis* populations.<sup>59, 394, 395</sup>

Several genotyping methods using different genetic markers (e.g., polymorphic GC-rich repetitive sequence, PGRS, or 16S and 23S rRNA-based methods) have been applied and tested for *M. tuberculosis* genotyping for the last three decades.<sup>400</sup> Nevertheless, only the three most widely used and consensual techniques employed in molecular epidemiology (Figure 1.12) are covered below. Still, when considering the choice of such method it is necessary to take in account that although a high discriminatory power is



**Figure 1.12** – *M. tuberculosis* chromosome representation and polymorphic loci used in molecular epidemiological methods. Legend: (a), *PvuII* restriction sites; (b), *IS6110*; (c) *IS6110* probe; (d), MIRUs. Adapted from Barnes *et al.*<sup>59</sup>

important to discriminate between epidemiologically non-related isolates, a discriminatory power too high can on the other end discriminate epidemiologically related isolates.<sup>54, 395</sup>

One of the earliest typing techniques for TB was the Restriction Fragment Length Polymorphism (RFLP), with *PvuII*, combined with a Southern hybridization using a partial sequence of the insertion sequence *IS6110* as a probe.<sup>401</sup> Insertion sequences are found dispersed throughout the bacterial chromosome in a variable number of copies and might be useful as probes to compare the number and size of the restriction fragment in which they can be found. *IS6110* has 1355 bp in length and is unique to the MTC.<sup>402</sup> Due to its polymorphic nature but relative stability in epidemiological related patients, the *IS6110* is also the genetic marker of choice for the RFLP-based typing method.<sup>118, 401, 403, 404</sup>

The RFLP-*IS6110* has a high discriminatory power but has, nevertheless, some drawbacks when compared with other available molecular typing techniques. RFLP-*IS6110* requires a considerably high amount of DNA (2 µg) that has to be extracted from cultured mycobacteria; for isolates with less than six *IS6110* copies, the discriminatory power becomes significantly

lower; it is of more difficult inter-laboratorial comparison; and, as it requires DNA extracted from culture it becomes a time-consuming technique.<sup>59, 395</sup>

RFLP-IS6110 was employed in the first molecular epidemiologic studies carried out during the 90s in the Lisbon Health Region that have identified a MDR-TB outbreak occurring mainly among HIV seropositive patients.<sup>26</sup> Genotyping by RFLP-IS6110 has also allowed the identification of a group of genetically close clusters that were first named as the A clusters and later, the Lisboa family.<sup>26</sup> Further cluster discrimination by *rpoB* mutations showed that independent transmission chains were linked with independent MDR-TB development followed by clonal expansion in the community.<sup>405</sup>

Another amply diffused typing method, because of its easy execution and because it is a PCR-based method, is Spoligotyping. This technique consists in the amplification of the spacer regions found within the DR (direct repeat) *locus*, which is composed by 36 bp spaced tandem repeats. The method consists in the amplification of these spacer regions and detection by reverse hybridization with specific probes for 43 spacer regions spotted onto a membrane followed by chemiluminescent detection.<sup>406</sup> This method will generate a hybridization profile for any clinical isolate. The spoligotyping profile can be expressed as a numerical profile (binary or octal code) which simplifies storing of typing data and greatly facilitates inter-laboratory comparison of typing data.<sup>55, 60</sup> Spoligotyping has nevertheless an inferior discriminatory power when compared with RFLP-IS6110 which can lead to transmission overestimates becoming, however, useful in discriminating RFLP-IS6110 clusters resulting from IS6110 low copy-number strains.<sup>407-409</sup> Moreover, multiple and independent spacer and DR deletions have been reported to lead to convergent evolution.<sup>410</sup>

The Beijing family of strains was initially described among patients from People's Republic of China and Mongolia as a group of genetically close strains that shared 2/3 of the IS-containing *PvuII* fragments but, having a identical spoligotype.<sup>70</sup> This highlights the usefulness of spoligotyping in tracking large and widespread genetic clades that exhibit some degree of divergence.

The most recent typing method is based on PCR amplification of different tandem repeat *loci* dispersed through the *M. tuberculosis* genome. These tandem repeat *loci* have a variable number of tandem repeats (VNTR) and are designated by Mycobacterial Interspersed Repetitive Unit (MIRU). As these *loci* are composed by small size tandem repeats (40-100 bp), the amplification and sizing of these *loci* allows the determination of the number of repeats present in each *locus* for each typed isolate, generating a numerical profile.<sup>411, 412</sup> The MIRU-

VNTR typing method started by including a set of 12 *loci*, which was subsequently increased up to 24 *loci* in order to achieve a higher discriminatory power. The discriminatory power of the 12-*loci* MIRU-VNTR has been reported as inferior to the one of RFLP-IS6110 but, however, the 15 *loci* set has been proposed as equivalent to RFLP-IS6110 and the 24-*loci* set as suitable for phylogenetic studies.<sup>54, 56, 411</sup> Additionally, MIRU-VNTR typing has the advantage of having a better discriminatory power when typing isolates with less than six IS6110 copies, *e.g.*, *M. bovis* isolates. Other advantages of the MIRU-VNTR typing method consist in the rapid turn-around time and, on the fact that only about 30 ng of DNA is necessary for the complete 24-*loci* typing. Using fluorochrome-tagged primers it is possible to analyse multiplexed amplicons by capillary electrophoresis and automate the sizing and allele assigning processes.<sup>57</sup>

Nowadays, MIRU-VNTR typing has almost completely replaced RFLP-IS6110 and it is considered the preferred typing method.

## 1.8 NEXT GENERATION SEQUENCING

The term “Next Generation Sequencing” (NGS) is commonly used to describe sequencing platforms based on more recent methodologies than the original sequencing based on Sanger chain termination chemistry or the chemical method from Maxam and Gilbert.<sup>413, 414</sup> While the original Sanger sequencing method required a specific primer to initiate reading through a template sequence, NGS strategy derives from the shotgun sequencing approach employed in the Human Genome Project.<sup>414, 415</sup> The new generation sequencing technologies are based on genomic fragmentation, adapter ligation and production of reads starting at random locations throughout the genome.<sup>415</sup> DNA templates are therefore sequenced in parallel in a stepwise iterative process in real-time in which each clonal template or single molecule is individually sequenced and analysed.<sup>415</sup> All NGS platforms end up by producing enormous amounts of data in a relatively short period and hence the reason that NGS is also associated with the term high-throughput sequencing.<sup>416</sup> Depending on the technology, the read length can vary as the first NGS technologies started by producing read lengths below the ones achieved by Sanger sequencing, although more recent technologies have surpassed the latter. Another important aspect to consider is coverage, *i.e.* the number of reads that overlap a given nucleotide position.

The first system to become available was from Roche, the GS-FLX 454 platform, and became available in 2004 using a combination of a sequencing-by-synthesis methodology known as

pyrosequencing and, single-molecule emulsion PCR.<sup>417</sup> Pyrosequencing is based on the detection of light from pyrophosphate groups released during dNTP incorporation.<sup>415</sup> The Illumina/Solexa Genome Analyzer was the second platform to become available and is presently the most widely used system.<sup>415, 418</sup> The Illumina platform uses a sequencing-by-synthesis methodology combining reversible chain termination and Bridge PCR for local cluster amplification.<sup>415</sup> Another widespread platform, the ABI SOLiD, uses a different approach for template sequencing by combining the emulsion PCR approach with a synthesis-by-ligation sequencing methodology using oligonucleotide probes.<sup>415, 419</sup>

These three platforms comprised the main second-generation sequencing technologies and are able to generate up to several billion bases per run.<sup>416</sup> Several updates have been made enabling higher read lengths.<sup>420</sup> Reads outputted by next generation sequencers can either be mapped to a reference genome, in a process known as reference assembly or, through finding overlapping regions be used without any reference genome in a *de novo* assembly process. Read length is an important factor as longer read lengths will in the future enable *de novo* assembly of more complex genomes.

A third generation of sequencing platforms is under development aimed at the development of sequencing methodology without using PCR for template amplification.<sup>416</sup> The third generation of sequencing technologies are based on a single-molecule sequencing system in which a read is derived from a single molecule and not from a clonal cluster.<sup>416</sup> Single-molecule systems have not only the advantage of minimizing errors that can be introduced by PCR but also to better reflect the relative abundance of template DNA. A shift in the detection method has also occurred among third-generation sequencers, from the more expensive optical imaging towards the detection by pH change in microstructures.<sup>420</sup> The Helicos HeliScope and the PacBio RS from Pacific Biosciences, are capable of single-molecule sequencing in real-time but still rely on fluorescence detection.<sup>420-423</sup> The Personal Genome Machine (PGM) sequencer from Ion Torrent/Life Technologies has successfully implemented single-molecule sequencing together with a non-optical detection system in a benchtop instrument.<sup>416, 420</sup> In the PGM, nucleotide incorporation is recorded by pH changes in a highly dense microwell array in which each well acts as a polymerization unit.<sup>424</sup> More recently, Ion Torrent/Life Technologies has released the Ion Proton Sequencer with a higher microwell density resulting in an increase in data output and allowing sequencing of a human genome on a chip.

The fourth generation of sequencers is expected to become available using nanopore technology, either biological or solid-state, that could generate long read lengths.<sup>420</sup> Solid-state

nanopores would be eventually more stable as it avoids the use of organic compounds.<sup>420</sup> Oxford Nanopore technologies are preparing to commercialize the first nanopore-based sequencer, the GridION system, that utilizes the heptameric protein  $\alpha$ -hemolysin as bionanopores for strand sequencing.<sup>425</sup> This system will be based on threading single-stranded DNA through the nanopores as nucleotide specific voltage changes are registered in real-time.<sup>420</sup> Further information released by Oxford Nanopore technologies point towards read lengths in the order of tens of kb. Comparison between the above covered sequencing technologies is outlined in Table 1.7.

**Table 1.7** – Comparison of the main NGS platforms representative of the different generations of sequencing technologies. Adapted from Niedringhaus *et al*<sup>420</sup> with updated information released by the manufacturers.

Generation	Company	Platform	Sequencing Method	Detection Method	Read Length (bp)
First	ABI/Life Technologies	3130xL – 3730xL	Capillary electrophoresis - Sanger	Fluorescence / Optical	600-1000
Second	Roche/454	GS FLX Titanium XL+ System	Pyrosequencing, sequencing by synthesis	Optical	Up to 1000
Second	Illumina	HiSeq2500	Reversible terminator, sequencing by synthesis	Fluorescence / Optical	2 x 100 (2 x 150 in Rapid Run Mode)
Second	ABI/SOLiD	5500xl W SOLiD System	Sequencing by ligation	Fluorescence / Optical	50 - 75
Second	Helicos	HeliScope	Single-molecule sequencing by synthesis	Fluorescence / Optical	25-35
Third	Pacific Biosciences	PacBio RS	Real-time, single-molecule DNA sequencing	Fluorescence / Optical	Up to 20 Kb (average 3-5 Kb)
Third	Ion Torrent / Life Technologies	Personal Genome Machine (PGM) sequencer	Sequencing by synthesis	pH change	Up to 400
Third	Ion Torrent / Life Technologies	Ion Proton	Sequencing by synthesis	pH change	Up to 200
Fourth	Oxford Nanopore	gridION	Nanopore strand sequencing	Electric Current	unknown

The use of NGS technologies to study the genomic diversity of *M. tuberculosis* is producing interesting data on the diversity, mode of evolution and host adaptation. Some studies have



employed the capability of next generation sequencing in studying the microevolution of *M. tuberculosis* in specific settings.<sup>426, 427</sup> Schurch *et al* have conducted a genome-wide analysis of two isolates, representing the first and the end isolate from a well studied transmission chain, and have found six different polymorphisms and an IS6110 transposition event. Further isolate characterization has suggested that the molecular evolution of *M. tuberculosis* in vivo is characterized by periods of relative genomic stability, followed by bursts of mutations<sup>428</sup>. Ford *et al* using the cynomolgous macaque model of infection studied the mutation rate of *M. tuberculosis* during latency by NGS found that *M. tuberculosis* acquires a similar number of chromosomal mutations during latency as occurs during active disease or in a logarithmically growing culture over the same period of time.<sup>429</sup>

Additionally, the ability to compare drug-resistant and susceptible strains with the same genotype identifies not only resistance-associated mutations but might also highlight compensatory mutations or others important in the process of drug resistance acquisition and physiological adaptation.<sup>430</sup>

## 1.9 OBJECTIVES

In Portugal, as already stated, TB constitutes a major public health concern, more specifically MDR-TB. It is in the two largest urban centers (Lisbon and Oporto) that this infectious disease associated with drug resistance phenotypes exerts its highest impact nationwide. In Lisbon, early studies carried out during the 1990s have shown that this situation is mostly due to the circulation of genetically close strains: the Lisboa family. An increasing knowledge of the molecular aspects of TB epidemiology can contribute to a better understanding of the dynamics of TB transmission and drug resistance development. This work is therefore based and covers the following:

- Genotypic analysis and molecular basis of resistance in MDR-TB isolates

In 2006, the designation of XDR-TB came into place to name what is now known to be an old problem associated with TB resistance to second-line drugs but, simultaneously imposed another question regarding TB control in Portugal: what is the prevalence of XDR-TB in the country and in the region?<sup>431, 432</sup> Moreover, as MIRU-VNTR genotyping method became available it also became important an updated genotypic analysis of MDR-TB clinical isolates and drug-resistance associated mutations.<sup>57, 412, 433</sup> The prevalence of specific drug resistance

mutations is of special importance for the implementation or development of rapid molecular tests. These research questions are addressed in Chapter 2 through a MIRU-VNTR genotypic analysis of MDR-TB strains recovered from Lisbon Health Region within a single year (2003) with the purpose of assessing the prevalence and clonality of circulating MDR-TB strains.<sup>24</sup>

Another important aspect that is focused throughout this work concerns the molecular basis of resistance. Since the prevalence of drug-resistance conferring mutations appear to vary geographically, it is highly important to know which mutations are responsible for drug resistance so that molecular resistance testing may be implemented in a setting-adapted manner. In Chapter 2 the main genes associated with resistance to first-line drugs were screened for mutations in order to establish the most prevalent mutations associated with drug resistance.

- Molecular determinants of resistance to second-line drugs and diversity of XDR-TB isolates

Following the previous line of research and given the high XDR-TB rate found previously, the analysis of genes associated with resistance to second-line drugs was also found to be important. The screening of *gyrA*, *rrs* and *tlyA* genes from XDR-TB isolates allied with MIRU-VNTR genotyping is described in Chapter 3.<sup>25</sup> Using this approach we intend to identify the major XDR-TB genetic clusters in Lisbon Health region as well as finding drug-resistant associated mutations that can be used as molecular markers for specific clusters.

- Laboratory perspective of the M/XDR-TB situation in Lisbon

Another objective, here delineated in the sequence of the previous studies, is to provide further evidence for a continued endemic M/XDR-TB situation in Lisbon, from laboratory data. In Chapter 4 the results from the compiled the records of 3 025 *M. tuberculosis* clinical isolates recovered between 2001-2006 are analysed in combination with MIRU-VNTR genotyping data from 100 isolates resistant to at least one first-line drug.

- Cross-resistance between second-line aminoglycosides

The lack of an apparent resistance determinant to KAN/AMK in Chapter 3 and a more recent report by Zaunbrecher *et al*<sup>372</sup> that associated *eis* gene overexpression with KAN resistance in *M. tuberculosis* reopened the question of the molecular basis of KAN resistance that we were unable to answer previously.<sup>434</sup> With the objective of gaining insight on the KAN/AMK cross-resistance mechanism we sought to characterize the promoter region of the *eis* gene in order

to find mutations that may exert a putative upregulatory effect in the strains previously tested and, strains isolated during the 90s outbreak known to belong to the Lisboa3 cluster (Chapter 5). Another aim of this study is to correlate hypothetical mutations to be found in the promoter region of the *eis* gene with the AMK/KAN resistance level.

- Contribution of *gidB* mutations to STP resistance and phylogeny

Given previously associations between *gidB* mutations and STP-resistance, it would be of interest to screen this gene for mutations that could correlate with STP-resistant isolates with no *rrs* or *rpsL* mutations and investigate the resistance levels.<sup>325, 326, 435</sup> The role of *gidB* gene and its contribution for STP resistance level by screening of STP-resistant and –susceptible isolates is addressed in Chapter 6. As *gidB* gene mutations have been proposed to be associated with specific phylogenetic lineages, any possible mutation-cluster association will also be investigated.

- Genomic distinctiveness and diversity of *M. tuberculosis* isolates in Lisbon, Portugal

The two major clusters associated with MDR-TB in Lisbon Health Region appear to be highly endemic to Portugal and, unique from a genotyping standpoint. The Lisboa family and the Q1 strains appear to show a high degree of success for transmission in the community, resulting in a high local prevalence (Chapters 2-4). Several questions emerge regarding these strains:

1. What is the phylogenetic positioning and/or phylogeographic origin of both strain types?
2. Do these strains possess any virulence factors already known or yet to discover?
3. What is the nature of its association with drug resistance and what are the microevolutionary events accompanying drug-resistance acquisition?

In an attempt to answer these questions and investigate the genomic diversity present in the MDR-TB strains circulating in the region, we sought to perform a comparative genomic analysis encompassing these strains and others circulating in Lisbon Health Region. Through a collaboration with the London School of Hygiene and Tropical Medicine and the King Abdullah University of Science and Technology, the genomes of 56 clinical isolates were sequenced by NGS technology, the great majority of which recovered in Lisbon Health Region. The first results from this comparative genomic analysis are described and discussed in Chapter 7.

Globally, all the data coming from these more concrete objectives and studies will contribute to a main purpose of gaining a better picture of MDR-TB epidemiology in Lisbon and towards

*M. tuberculosis* biology and adaptation in this host population. This approach can ultimately contribute to the deployment of effective measures to control MDR-TB.

# **Multidrug-Resistant Tuberculosis in Lisbon, Portugal: A Molecular Epidemiological Perspective**

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## 2.1 ABSTRACT

Portugal has the fourth highest TB incidence rate of the European Union. Thirty-nine percent of all cases originate in Lisbon Health Region. Portugal also presents high levels of MDR-TB (1.1%, primary rate and 6.7%, in retreatment cases). In the present study we have characterized 58 MDR-TB clinical isolates by: i) determining the resistance profile to first and second-line drugs used in the treatment of tuberculosis; ii) genotyping all isolates by MIRU-VNTR; iii) analyzing mutations conferring resistance to INH, RIF, STP and PZA, in *katG*, *mabA-inhA*, *rpoB*, *rpsL*, *rrs* and *pncA* genes. We have therefore established the prevalence of the most common mutations associated with drug resistance in the Lisbon Health Region; C-15T in *mabA-inhA* for INH; S531L in *rpoB* for RIF; K43R in *rpsL* for STP; and V125G in *pncA* for PZA. By genotyping all isolates and combining with the mutational results we were able to assess the isolates genetic relatedness and determine possible transmission events. Strains belonging to family Lisboa, characterized several years ago, are still responsible for the majority of the MDR-TB. Even more alarming is the high prevalence of extensive drug resistant tuberculosis (XDR-TB) among the MDR-TB isolates, which was found to be 56%. The TB status in Portugal therefore requires urgent attention in order to contain the strains continuously responsible for MDR-TB and now, XDR-TB.

## 2.2 INTRODUCTION

Portugal has the fourth highest tuberculosis (TB) incidence rate in the European Union (EU), having registered in 2004, 33.7 cases per 100 000 habitants nationwide. Although this rate has diminished in the last 20 years to less than a half, the decline rate has been very slow, having decreased only 2.7 cases per 100 000 people from 2003 to 2004.<sup>436</sup>

In the year of 2004, 39% of all new cases reported nationwide originated from Lisbon Health Region. The majority of these cases (72%) were concentrated in Lisbon district, of which 74% were associated with three high-risk groups for TB, namely, human immunodeficiency virus (HIV) infected, drug users and immigrants.<sup>436</sup>

The emergence of MDR-TB, that is, resistance to at least INH and RIF, poses a serious threat, not only to national tuberculosis control plans, but also to the implementation of an effective directly observed short-course treatment (DOTS) strategy. Portugal also reports high rates of MDR-TB. In 2004, Portuguese Health authorities have reported a rate of primary MDR-TB of 1.1%, and 6.7% among retreatment cases.<sup>436</sup>

Drug resistance generally occurs as a result of a regimen with the wrong combination of drugs or, due to the patient lack of adherence to the regimen. The only known mechanism for resistance development by *M. tuberculosis*, is through mutation acquisition in chromosomal genes associated with drug resistance. Several genes have been associated with drug resistance, namely *katG*, *inhA*, *kasA* and *ahpC*, with INH;<sup>207, 437, 438</sup> *rpoB* with RIF resistance;<sup>250, 438, 439</sup> *rpsL* and *rrs* with STP resistance;<sup>311, 313, 440</sup> *embCAB* with EMB resistance;<sup>278, 284, 289</sup> and *pncA* with PZA resistance.<sup>332, 336, 340, 347, 441</sup> The emergence of MDR-TB strains is due to sequential acquisition of mutations in some of the genes above.

Epidemiological surveys concerning MDR-TB strains are necessary to gain insight into the transmission and resistance development mode of TB, and ultimately to manage properly the local TB situation. Strain typing is an invaluable process in epidemiology and, the last years epidemiological surveys have benefited from the development of additional molecular epidemiological methods, alternative to the traditional Restriction Fragment Length Polymorphism (RFLP) followed by hybridization with Insertion Sequence (IS) 6110.<sup>59, 403</sup> Such an example is the use of MIRU-VNTR for strain typing. MIRU-VNTR typing is based on the determination of the number of repeating units on several chromosomally dispersed *loci*.<sup>57, 59,</sup>

<sup>394</sup> Although the discriminative power reported by some studies is lower than that of RFLP-



IS6110, it is not as time-consuming as the former, facilitates inter-laboratorial data exchange, and is less likely to underestimate the recentness of transmission, as reported for RFLP-IS6110.<sup>54, 442</sup>

In the present study, we have characterized several resistance-associated genes from 58 MDR-TB isolates recovered from Lisbon Health Region and, assessed the genetic relatedness of these isolates by MIRU-VNTR. As the emergence of more deadly strains of *M. tuberculosis* occur throughout the world we have assessed the predominance of strains with extensive drug resistance (XDR-TB, *i.e.*, MDR-TB plus resistance to at least one injectable second-line drug and a FQ) among the studied isolates.<sup>432, 443, 444</sup> Since mutations conferring resistance varies geographically, the characterization of the most prevalent mutations in a given community allows the development of locally adapted, resistance molecular screening tests. Allaying the determination of resistance-associated mutations with strain typing by MIRU-VNTR not only allows us to gain knowledge concerning the mode of resistance acquisition, but also permit a more accurate characterization of TB transmission mode in the community, and the identification of possible MDR-TB outbreaks.

## 2.3 MATERIALS AND METHODS

### Clinical Isolates and DNA

Fifty-eight MDR-TB isolates were recovered from patients whose clinical samples were sent for testing at the mycobacteriology unit at the National Institute of Health Dr. Ricardo Jorge (INSA-RJ), Lisbon. The isolates were collected during the year of 2003 and are a subset of 116 MDR-TB strains isolated in the same year. Isolates were cultured on LJ slants or, in the BACTEC 960 (Becton Dickinson™) system and then passed to LJ slants. Identification of *M. tuberculosis* complex was accomplished using the Accuprobe (Gen-Probe™) method.

DNA of the above isolates was extracted as previously described by van Soolingen *et al.*<sup>445</sup>

Isolates were tested for first-line drug susceptibility by the BACTEC 960™ MGIT™ methodology for INH (0.1 mg/L), RIF (1.0 mg/L), STP (1.0 mg/L), EMB (5.0 mg/L) and PZA (100 mg/L), according to the manufacturer's instructions. Isolates resistant to at least INH and RIF were considered MDR-TB and, tested for second-line drug susceptibility by the radiometric BACTEC

460™ system to CAP (1.25 mg/L), AMK (1.0 mg/L), KAN (5.0 mg/L), CIP (1.0 mg/L), OFX (2.0 mg/L), ETH (5.0 mg/L) and PAS (4.0 mg/L), according to Pfyffer *et al.*<sup>446</sup>

## MICs

PZA minimum inhibitory concentration was determined using concentrations of 100, 300 and 900 mg/L by the BACTEC 960 MGIT system. Minimum inhibitory concentrations were defined as, the minimum tested drug concentration that rendered a susceptible result.

## Gene amplification and sequencing

Several resistance associated genes from MDR-TB clinical isolates have been amplified and characterized according to the respective resistance pattern. We have characterized the *katG* gene and the *mabA-inhA* operon regulatory region, in clinical isolates resistant to INH; *rpoB* resistance determinant region (RRDR) in RIF resistant isolates; *rpsL* and *rrs* for STP resistant isolates; and *pncA* for PZA resistant isolates. All amplification reactions were carried out using PuReTaq™ Ready-To-Go™ PCR beads (GE Healthcare™), with 10 pmol of each oligonucleotide primer used and approximately 50 ng of template DNA, accordingly to the manufacturer's instructions.

A 620 bp internal fragment (904-1523) of *katG* gene was amplified by polymerase chain reaction (PCR) with forward oligonucleotide primer katG904 and reverse oligonucleotide primer katG1523 (Table 2.1). The cycling conditions consisted in an initial denaturing step of two minutes at 94°C, followed by 50 cycles of amplification (denaturation at 94°C for 1min; annealing at 60°C for 1 min; and extension at 72°C for 1 min).

The *mabA-inhA* regulatory region was amplified in a 248 bp fragment (positions -168 to 80, relative to *mabA-inhA* initiation codon) using forward oligonucleotide primer mabA-inhA-F and reverse oligonucleotide primer mabA-inhA-R (Table 2.1). Cycling conditions for this amplification consisted in an initial 5 min denaturation step; followed by 30 cycles of denaturation at 94°C for 5 min, annealing at 65°C for 1 min, and an extension step at 72°C for 1,5 min; a final extension step at 72°C for 10 min was performed.

A 409 bp fragment of *rpoB* (GenBank accession no. L27989) encompassing the RIF resistance-determining region was amplified by PCR with forward oligonucleotide primer TR1 and, reverse oligonucleotide primer RPOB-2 (Table 2.1). The cycling conditions consisted in a denaturation step at 94°C for 4 min, followed by 30 cycles of amplification (denaturation at

94°C for 1 min, primer annealing at 60°C for 1 min, and extension at 72°C for 1 min), and a last final extension step at 72°C for 15 min.

**Table 2.1**– Oligonucleotide primers used to amplify and sequence the studied genes.

	Sequence	T <sub>m</sub> (°C)	Reference
katG904	5'-AGCTCGTATGGCACCAGAAC-3'	68,4	Uhl <i>et al</i> <sup>447</sup>
katG1523	5'-TTGACCTCCCACCCGACTTG-3'	69,5	Uhl <i>et al</i> <sup>447</sup>
mabA-inhA-F	5'-CCTCGCTGCCAGAAAGGGA-3'	66,6	Coll <i>et al</i> <sup>448</sup>
mabA-inhA-R	5'-ATCCCCCGGTTTCCTCCGGT-3'	66,6	Coll <i>et al</i> <sup>448</sup>
PNCA-2R	5'-GAACACCGCCTCGATTGCCG-3'	74.0	Hannan <i>et al</i> <sup>449</sup>
PNCA-8	5'-GGTTGGGTGGCCGCCGGTCAG-3'	80.2	Hannan <i>et al</i> <sup>449</sup>
PNCA-6	5'-CCTCGTCGTGGCCACCGC-3'	75.0	Hannan <i>et al</i> <sup>449</sup>
PNCA-10	5'-GCTGGTCATGTTCGCGATCG-3'	71,3	Hannan <i>et al</i> <sup>449</sup>
PNCA-11	5'-GCTTTGCGGCGAGCGCTCCA-3'	78.6	Hannan <i>et al</i> <sup>449</sup>
RPOB-1	5'-GGGAGCGGATACACCC-3'	73.0	Portugal <i>et al</i> <sup>405</sup>
RPOB-2	5'-GCGGTACGGCGTTTCGAT-3'	73.1	Portugal <i>et al</i> <sup>405</sup>
RPSL-1	5'-GGCCGACAAACAGAACGT-3'	63.8	Kirschner <i>et al</i> <sup>450</sup>
RPSL-2	5'-GTTCACCAACTGGGTGAC-3'	58.8	Kirschner <i>et al</i> <sup>450</sup>
RRS-1	5'-GAGAGTTTGATCCTGGCT-3'	62.9	Kirschner <i>et al</i> <sup>450</sup>
RRS-2	5'-TGCACACAGGCCACAAGG-3'	72.5	Kirschner <i>et al</i> <sup>450</sup>
TR1	5'-TACGGTCGGCGAGCTGAT-3'	71.7	Portugal <i>et al</i> <sup>405</sup>

The entire open reading frame (ORF) of *rpsL* (GenBank accession no. L08011.1), plus 46 nucleotides upstream and 86 nucleotides downstream, were amplified by PCR in a 504 bp fragment using forward oligonucleotide primer RPSL-1, and reverse oligonucleotide primer RPSL-2 (Table 2.1). Cycling conditions for this amplification consisted in 40 cycles of a denaturation step at 94°C for 1 min, a primer annealing step at 57°C for 2 min and, an extension step at 72°C for 2 min.

The region comprehended by nucleotides 10 to 1037 in *rrs* was amplified by PCR using forward oligonucleotide primer RRS-1 and reverse oligonucleotide primer RRS-2 (Table 2.1). The cycling conditions consisted in 40 cycles of a denaturation step at 94°C for 1 min, a primer annealing step at 60°C for 2 min and, an extension step at 72°C for 2 min.

The entire *pncA* ORF, as well as 124 bp upstream and 59 bp downstream, was amplified in a 744 bp fragment by PCR, using forward oligonucleotide primer PNCA-11 and, reverse oligonucleotide primer PNCA-8 (Table 2.1). Cycling conditions consisted in: a denaturation step at 94°C for 5 min; 20 cycles of denaturation at 94°C for 30 sec, primer annealing at 68°C for 30 sec, with a decrease of 0.5°C per cycle and, extension at 72°C for 30 sec; 15 cycles of

denaturation at 94°C for 30 sec, primer annealing at 58°C for 30 sec and, extension at 72°C for 30 sec; finally an extension step at 72°C for 8 min.

Amplicons were purified with MicroSpin™ S-300 HR Columns (GE Healthcare™) prior to sequencing. Sequencing reaction was performed with BigDye™ Terminator Cycle Sequencing Kit with AmpliTaq™ DNA polymerase (Applied Biosystems™) using oligonucleotide primers katG904 for *katG* amplicons; mabA-inhA-F for *mabA-inhA* regulatory region amplicons; RPOB-1 for *rpoB* amplicons; RPSL-1 for *rpsL* amplicons; RRS-1 and RRS-2 for *rrs* amplicons; and, PNCA-6, PNCA-10, PNCA-11 and PNCA-2R for *pncA* amplicons (Table 2.1). Obtained sequences, were aligned with the respective wild-type alleles, from *M. tuberculosis* H37Rv, using CLC Free Workbench (CLC Bio™, Aarhus C, Denmark) and analyzed using BioEdit (v.7.0.5.2, T.A. Hall) software.

### Endonuclease digestions

To rapidly detect some resistance-associated mutations, RFLP analysis was used. Mutations S315T and R463L, in *katG*, were detected by *MspI* (New England Biolabs™) digestion of the *katG* amplicon. Two micrograms of PCR product were digested with 10U of *MspI* in accordance with the manufacturer's instructions for 1 h at 37°C. The analysis of the restriction profile allows detection of mutations G944C (S315T) and G1388T (R463L) as described by Uhl *et al.*<sup>447</sup>

Mutation A130G in *rpsL* was detected by *MboII* (New England Biolabs™) digesting of the *rpsL* amplicon. Two micrograms of PCR product were digested with 5U of *MboII* accordingly to the manufacturer's instructions for 1 h at 37°C. Absence of restriction indicates the presence of mutation A130G.

Mutations T359C and T374G in *pncA* were detected by *SacII* and *BtgZI* (New England Biolabs™) digestion, respectively. Digestions were done independently and sequentially using approximately 1 µg of PCR product, with 10 U of *SacII* or with 1 U of *BtgZI*, for 1 h at 37°C for *SacII*, or 60°C for *BtgZI* as recommended by the manufacturer. Occurrence of restriction indicates the presence of the tested mutation.

### MIRU-VNTR genotyping

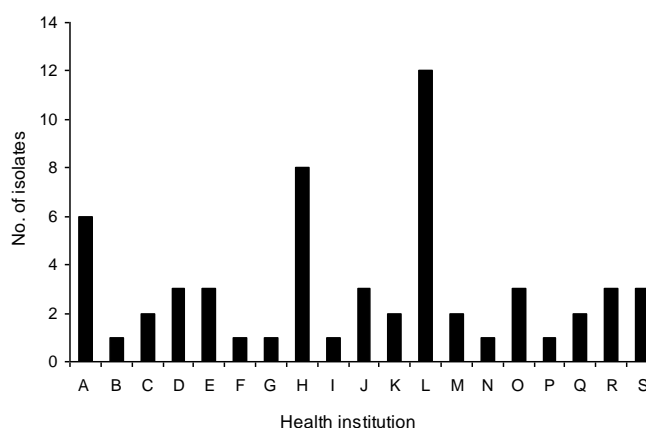
MIRU-VNTR genotyping was performed for each isolate by multiplex PCR amplification of 12 MIRU-VNTR *loci* using HotStarTaq™ DNA polymerase (Qiagen™), as previously described by Supply *et al.*<sup>57</sup>

## Clustering analysis

Isolates were clustered accordingly to their MIRU-VNTR profile using BioNumerics software (Applied Maths™, Sint-Martens-Latem, Belgium). Pearson coefficient was used to calculate the similarity matrix and clustering was performed by the unweighted pair group method with arithmetic mean (UPGMA).

## 2.4 RESULTS

**Multidrug resistance.** In 2003, 116 MDR-TB isolates corresponding to the same number of patients, from 22 different hospital units across Lisbon's Health Region, were isolated or received for testing at the Portuguese National Institute of Health – Dr. Ricardo Jorge's Unit of Mycobacteriology. Of these 116 isolates, 58 have been fully characterized since the remaining isolates had lost viability. These 58



isolates originated from 19 different health institutions units across Lisbon's Health Region (Figure 2.1), herein designated as health institutions A to S. Among these isolates, six different resistance patterns to first-line antibacillary drugs were observed (Table 2.2); resistance to all first-line antibacillary drugs was the most common pattern.

**Table 2.2** – Resistance patterns to first-line drugs observed in the studied isolates, I, INH; R, rifampicin; S, streptomycin; E, ethambutol; P, pyrazinamide.

Resistance pattern	No. of isolates (%)
IR	2 (3.4)
IRP	5 (8.6)
IRS	5 (8.6)
IRSE	3 (5.2)
IRSP	16 (27.6)
IRSEP	27 (46.6)
<b>Total</b>	<b>58</b>

**Resistance Associated Mutations.** We started the characterization of the 58 MDR-TB isolates by determining the molecular basis of resistance, accordingly to the resistance pattern exhibited by each. INH-resistant isolates were screened for mutations at the *katG* gene and *mabA-inhA* regulatory region; RIF resistance-determining region (RRDR) of *rpoB* gene was characterized by sequencing in RIF-resistant isolates; *rpsL* gene and STP resistance-determining region in *rrs* gene were characterized by PCR-RFLP and sequencing in STP-resistant isolates; finally, the *pncA* gene and its putative promoter have been characterized by PCR-RFLP and sequencing in PZA-resistant isolates.

In the characterization of resistance to INH we started by characterizing an internal region of *katG* gene, associated with resistance in previous studies.<sup>437, 448</sup> We found that seven isolates displayed a serine-to-threonine substitution at codon 315, while the remaining isolates did not exhibit any mutation at the referred region (Table 2.3). All 58 isolates were also screened for mutations in the *mabA-inhA* regulatory region; 53 (91.4%) isolates had a cytosine-to-thymine substitution at position -15, and the remaining five did not exhibit any mutation in the same region.

Mutations conferring resistance to RIF were found in 57 (98.3%) out of 58 isolates resistant to RIF. We have found only 2 different mutations in the RRDR (Table 2.3), all of which previously associated with RIF resistance. Fifty-three isolates had a serine-to-leucine substitution at codon 531 and 4 isolates had an aspartic acid-to-valine substitution at codon 516 of RpoB protein.

The screening for mutations conferring resistance to STP in *rpsL* and *rrs* among the 51 STP-resistant isolates, resulted in the identification of only one mutation – a lysine-to-arginine change at codon 43 of the RpsL protein, detected in 34 STP-resistant isolates (Table 2.3). The remaining 17 (33,3%) isolates didn't have any mutation at the *rpsL* gene or at the *rrs* STP resistance-determining region.

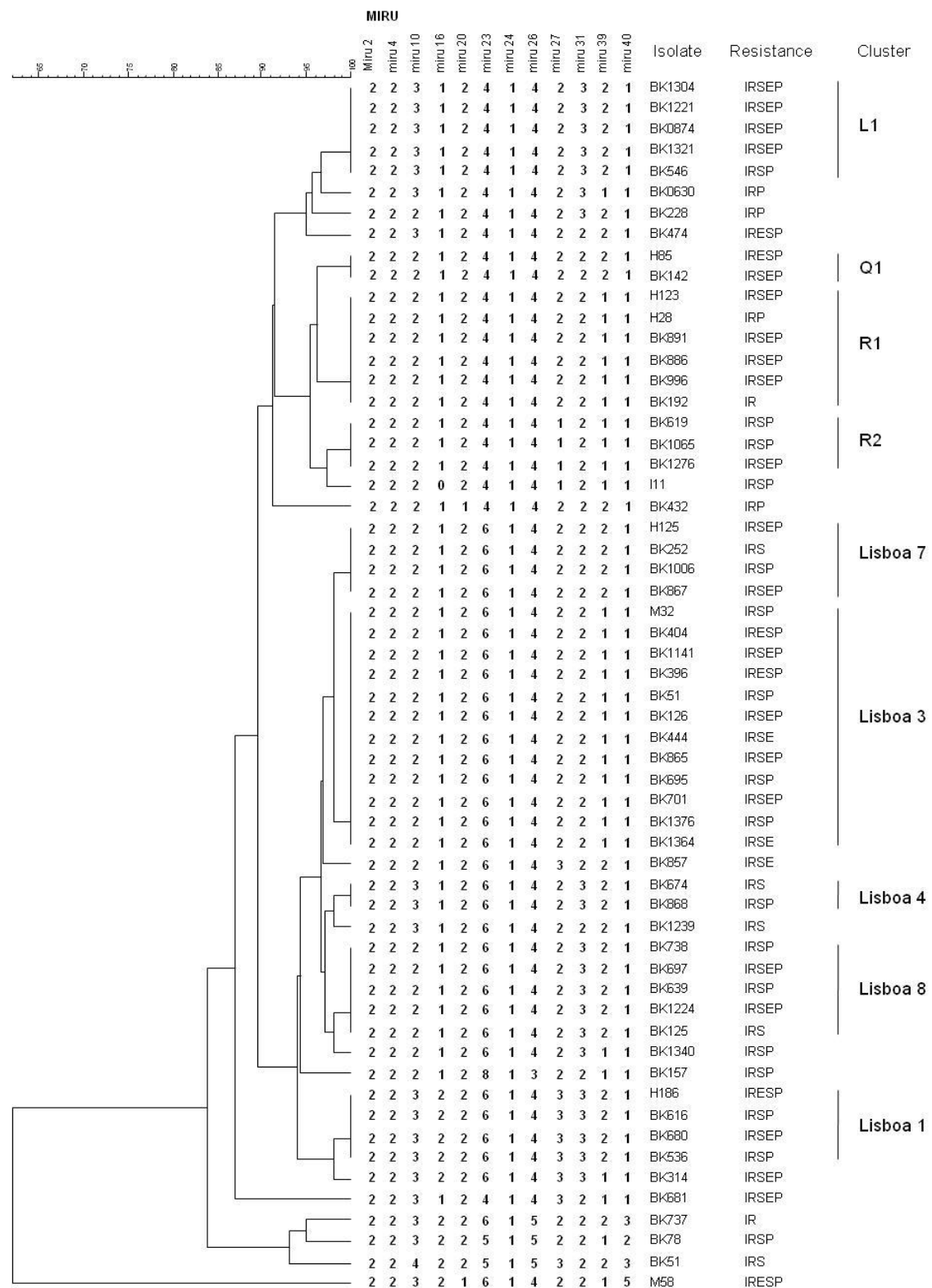
Twelve different mutations in the *pncA* gene were found among the 48 PZA-resistant isolates, the most common being a thymine-to-cytosine and a thymine-to-guanine substitution at nucleotides 359 and 374, respectively (Table 2.3). Three new mutations were identified in the course of this study, a thymine insertion at nucleotide position 282; a 9 bp deletion at nucleotide position 381; and a cytosine-guanine insertion at nucleotide position 439. All the PZA-resistant isolates had a mutation in the *pncA* gene. Mutations 282InsT and 381Del9bp were associated with medium- (>300 mg/L) and high- (>900 mg/L) level resistance, respectively.

**Table 2.3** – Mutations found in resistance-associated genes of the 58 studied clinical isolates.

Gene	Mutation (nt)	Mutation (aa)	No. of Isolates (%)
<i>katG</i>	None	None	51(87.9)
	G944C	S315T	7(12.1)
	<b>Total</b>		<b>58</b>
<i>inhA</i>	None	None	5(8.6)
	C-15T		53(91.4)
	<b>Total</b>		<b>58</b>
<i>rpoB</i>	None	None	1(1.7)
	C2431T	S531L	53(91.4)
	A2386T	D516V	4(6.9)
	<b>Total</b>		<b>58</b>
<i>rpsL</i>	None	None	17(33.3)
	A130G	K43R	34(66.7)
	<b>Total</b>		<b>51</b>
<i>pncA</i>	T2C	M1T	7(14.6)
	A226C	T76P	1(2.1)
	250InsC	Frameshift	3(6.3)
	282InsT	Frameshift	1(2.1)
	C285A	Stop	1(2.1)
	T359C	L120P	11(22.9)
	T374G	V125G	19(39.6)
	381Del9bp	Frameshift	1(2.1)
	392InsG	Frameshift	1(2.1)
	T398C	I133T	1(2.1)
	T416G	V139G	1(2.1)
	439InsCG	Frameshift	1(2.1)
	<b>Total</b>		<b>48</b>

**MIRU-VNTR Clusters.** All 58 isolates were genotyped by MIRU-VNTR and grouped in clusters, that is, isolates with 100% similarity index, accordingly to the similarity of their MIRU-VNTR profile. Forty-three (74.1%) isolates were grouped in nine clusters, which suggests a recent high-transmission rate (Figure 2.2). Families were designated and named accordingly to previous Lisbon Health Region's RFLP-IS6110 typing results.<sup>26</sup>

The most predominant family is family Lisboa, initially identified by Portugal *et al*<sup>26</sup> as Clusters A, containing 32 (55.2%) isolates and 5 of the 9 clusters identified in this study.



**Figure 2.2** – MIRU-VNTR dendrogram of the isolates characterized in the present study.

Resistance profile: I, isoniazid; R, rifampicin; S, streptomycin; E, ethambutol; P, pyrazinamide.



One of the clusters of the Lisboa family, named Lisboa3, has also been identified in previous studies by some of us as the most predominant cluster, which allowed the detection of at least two independent outbreaks.<sup>405</sup> In this study, Lisboa3 remains the most predominant cluster of isolates: 12 (20.7%) isolates originating from 10 different hospital units belong to this cluster.

**Cluster's mutational analysis.** Combining a cluster analysis with a mutational analysis of a given cluster allows the discrimination of that cluster, permitting the differentiation of independent and more recent transmission events.<sup>405, 451</sup> Results are presented in Table 2.4.

In cluster R2 one of the isolates differs from the remaining isolate by having additional resistance to EMB; such, points to a common origin but a new strain has already arose in this cluster. Cluster R1 although constituted by 6 clinical isolates has 5 different strains. Isolates H123 and BK891 probably represent the same strain since they share the same resistance and mutational profile. Cluster Q1 is constituted by only 2 isolates that probably represent the same strain. Cluster L1 has one different isolate that acquired resistance to EMB. Cluster Lisboa7 isolates correspond to different strains since there isn't a common resistance and mutational profile. Cluster Lisboa3 is composed by 12 clinical isolates from 10 different hospital units. Several strains are represented in this cluster; isolates BK404, BK1141, BK126 and BK1376 share the same mutational profile, isolate BK1376 differs from the remaining by lacking resistance to EMB. The remaining Lisboa clusters present similar situations as the Lisboa7 cluster, in which each isolate represents a different strain since there is no common mutational and resistance profile among isolates.

Nosocomial transmission does not seem to be significant, as only two identical isolates in one hospital and another two in another hospital were detected.

Analysing the mutations present in a given cluster, also allows an insight on the mode of resistance acquisition in the given family or cluster. We have found that mutation K43R in RpsL protein is present in all isolates of Lisboa family. Analysing Lisboa3 cluster we can observe that, probably, resistance to STP was the first to be acquired, since mutation K43R is common to all isolates, followed by acquisition of resistance to RIF, then INH and last to PZA or EMB.

**Table 2.4** – Clustered isolates mutational and origin characterization.

Cluster	Isolate	Origin	Resistance	InhA	KatG	RpoB	RpsL	PncA
R2	BK619	L	IRSP	C-15T	none	S531L	none	T374G
R2	BK1065	E	IRSP	C-15T	none	S531L	none	T374G
R2	BK1276	E	IRSEP	C-15T	none	S531L	none	T374G
R1	H123	H	IRSEP	C-15T	none	S531L	none	T374G
R1	H228	H	IRP	C-15T	none	S531L	-	T374G
R1	BK891	E	IRSEP	C-15T	none	S531L	none	T374G
R1	BK886	R	IRSEP	C-15T	none	none	none	T374G
R1	BK996	D	IRSEP	C-15T	none	S531L	K43R	T359C
R1	BK192	J	IR	none	S315T	S531L	-	-
Q1	H85	H	IRSEP	C-15T	none	S531L	none	T374G
Q1	BK142	H	IRSEP	C-15T	none	S531L	none	T374G
L1	BK1304	A	IRSEP	C-15T	none	S531L	none	T374G
L1	BK1221	A	IRSEP	C-15T	none	S531L	none	T374G
L1	BK874	J	IRSEP	C-15T	none	S531L	none	T374G
L1	BK1321	R	IRSEP	C-15T	none	S531L	none	T374G
L1	BK546	R	IRSP	C-15T	none	S531L	none	T374G
Lisboa7	H125	H	IRSEP	C-15T	none	S531L	K43R	439InsCG
Lisboa7	BK252	Q	IRS	C-15T	none	S531L	K43R	-
Lisboa7	BK1006	S	IRSP	C-15T	none	S531L	K43R	T359C
Lisboa7	BK867	L	IRSEP	C-15T	none	S531L	K43R	T359C
Lisboa3	M32	M	IRSP	C-15T	S315T	D516V	K43R	250InsC
Lisboa3	BK404	H	IRSEP	C-15T	none	S531L	K43R	T2C
Lisboa3	BK1141	A	IRSEP	C-15T	none	S531L	K43R	T2C
Lisboa3	BK396	L	IRSEP	None	S315T	S531L	K43R	T416G
Lisboa3	BK51	P	IRSP	C-15T	none	S531L	K43R	T359C
Lisboa3	BK126	K	IRSEP	C-15T	none	S531L	K43R	T2C
Lisboa3	BK444	F	IRSE	C-15T	none	S531L	K43R	-
Lisboa3	BK865	L	IRSEP	C-15T	S315T	S531L	K43R	T359C
Lisboa3	BK695	B	IRSP	C-15T	none	S531L	K43R	282InsT
Lisboa3	BK701	L	IRSEP	C-15T	none	S531L	K43R	T359C
Lisboa3	BK1376	O	IRSP	C-15T	none	S531L	K43R	T2C
Lisboa3	BK1364	N	IRSE	C-15T	none	S531L	K43R	-
Lisboa8	BK738	L	IRSP	C-15T	none	S531L	K43R	T2C
Lisboa8	BK697	S	IRSEP	C-15T	none	S531L	K43R	T359C
Lisboa8	BK639	S	IRSP	C-15T	none	S531L	K43R	T374G
Lisboa8	BK1224	D	IRSEP	C-15T	none	S531L	K43R	T398C
Lisboa8	BK125	G	IRS	C-15T	none	S531L	K43R	-
Lisboa4	BK674	C	IRS	C-15T	none	S531L	K43R	-
Lisboa4	BK868	L	IRSP	C-15T	none	S531L	K43R	T359C
Lisboa1	H186	H	IRSEP	C-15T	none	S531L	K43R	T2C
Lisboa1	BK616	L	IRSP	C-15T	none	S531L	K43R	392InsG
Lisboa1	BK680	L	IRSEP	C-15T	none	S531L	K43R	T359C
Lisboa1	BK536	O	IRSP	C-15T	none	S531L	K43R	250InsC

**XDR-TB.** In order to determine the resistance profile for second-line drugs we were able to perform drug susceptibility tests to ETH, PAS, CAP, KAN, AMK, CIP and OFX in 50 of the 58 studied isolates. We have obtained 11 different resistance patterns (Table 2.5), of which six fit into the current XDR-TB definition.<sup>432</sup> In total, 28 (56%) of the 50 MDR-TB isolates tested were also XDR-TB and, 32 (64%) were resistant to at least three classes of second-line drugs.

**Table 2.5** - Second-line drug resistance profiles found among 50 MDR-TB isolates; CAP, capreomycin; AMK, amikacin; KAN, kanamycin; CIP, ciprofloxacin; OFX, ofloxacin; ETH, ethionamide; PAS, para-amino salicylic acid.

Resistance Pattern	No. of isolates (%)
CAP AMK ETH PAS	1(2)
CAP AMK KAN CIP OFX ETH	7(14)
CAP AMK KAN ETH	2(4)
CAP AMK KAN ETH PAS	1(2)
CAP AMK KAN OFX	1(2)
CAP CIP OFL ETH	2(4)
CAP KAN CIP OFX ETH	10(20)
CAP KAN CIP OFX ETH PAS	5(10)
CIP OFX ETH	4(8)
ETH	11(22)
KAN CIP OFX ETH	3(6)
Pan-susceptible	3(6)
<b>Total</b>	<b>50</b>

## 2.5 DISCUSSION

In the present study we have genotyped 58 MDR-TB isolates recovered in the same year, by MIRU-VNTR, and characterized the mutations associated with drug resistance in each isolate. This would serve as a starting point not only to evaluate the prevalence of the mutations associated with resistance, but also to confirm cluster integrity in order to give a more accurate vision of the transmissibility of the above strains. These 58 MDR-TB isolates, a subset of 116 MDR-TB clinical isolates, were detected by testing 1 053 isolates for drug susceptibility. This, points to a MDR-TB prevalence rate of 11% among isolates from Lisbon Health Region, which is significantly higher than the MDR-TB prevalence rate officially reported by Portuguese health authorities in the same year for the districts comprehended by this region (3.0%, 1.7% and 4.4% for Lisboa, Santarém and Setúbal districts respectively).<sup>452</sup> Moreover, only 17 MDR-TB cases were reported for the entire country by Portuguese health authorities in 2003, while we have isolated 116 MDR-TB isolates (each corresponding to a case) only from Lisbon health region in the same year. The MDR-TB status in Lisbon health region and in the entire country may therefore be underestimated.<sup>453</sup>

We have characterized the mutations associated with resistance to INH, RIF, STP and PZA. Unexpectedly the most prevalent mutation found conferring resistance to INH occurred in the promoter region of *mabA-inhA*. This is in contradiction with published studies, where the most prevalent mutation found is in *katG*.<sup>226-229, 232, 454, 455</sup> Mutation C-15T in *inhA*, which was detected in 53 (91.4%) isolates, has been considered a hypermorphic mutation, resulting in higher levels of InhA.<sup>456</sup> Overexpression of *inhA* has also been associated with INH resistance, and more recently Vilch  ze *et al* have resolved the primary target of INH as being InhA.<sup>206, 238</sup> Nevertheless, overexpression of *inhA* was also associated with ETH resistance, an INH second-line drug analogue that shares with the former part of its action mechanism.<sup>207, 238</sup> The fact that the studied isolates present a high prevalence of a mutation that causes overexpression of *inhA*, may also account for the high ETH resistance prevalence (92%).

Mutations in RRDR region of *rpoB* leading to resistance to RIF were detected in 57 (98.3%) isolates, while the remaining isolate had a wild-type RRDR, which is in accordance with previous results.<sup>250, 439</sup> Mutation S531L was present in 53 (91.4%) isolates, being the most prevalent mutation associated with RIF resistance. Such prevalence rate is significantly higher than the prevalence rates reported in previous studies, which varied between 29% and 57%, in clinical isolates or *in vitro* generated mutants.<sup>250, 439, 457, 458</sup> However, the high prevalence of mutation S531L cannot be explained as a result of character ancestry, since isolates displaying this mutation originated from several diverse clusters, not exclusively associated with MDR-TB or even any resistance pattern (data not shown) and, in the same cluster other mutations than S531L may exist.

We have screened the STP-resistant isolates included in this study, for mutations conferring resistance to STP in *rpsL* and *rrs* genes. However, we have only found one mutation (K43R in RpsL) previously associated with STP resistance.<sup>311, 313, 323, 459</sup> Since the remaining isolates (33.3%) did not exhibit any mutation in *rpsL* and *rrs* genes, the resistance phenotype may be due to alterations in the permeability to the drug as previously reported.<sup>313</sup> Another factor that could account to the absence of mutations in these STP-associated genes may be related to the critical concentration of STP used in the DST test. The critical concentrations used in this study are in accordance with those recommended by the system manufacturer's instructions. However, such concentration may not be adequate for testing the studied population, resulting in false-resistant isolates (classified as major errors).

Genetic alterations conferring PZA resistance occur mainly in the pyrazinamidase/nicotinamidase encoding gene (*pncA*), although PZA-resistant clinical isolates

with a wild-type *pncA* gene have been reported, no other genetic mechanism of resistance is currently known for PZA.<sup>348, 350, 440, 441</sup> All PZA-resistant isolates included in this study exhibited mutations in the *pncA* open-reading frame. Three of the frameshift mutations found have never been previously described; two were associated with medium- (282InsT; >300 mg/L) and high-level (381Del9bp; >900 mg/L) resistance. Even though a wide variety of mutations exist, several authors have reported a clustering of these mutations around three regions, codon positions 3 to 17, 61 to 85 and 132 to 142.<sup>332, 346, 350</sup> Of the mutations obtained in the present study, only four represented by seven isolates (14.7%), are localized in these regions or in the proximity.

Although some commercial molecular methods for the detection of resistance already exist, the predominance of mutations conferring drug resistance varies geographically. The knowledge of the prevalence of resistance-associated mutations may therefore allow the development of in-house methods, locally adapted for the early detection of resistance.<sup>292, 332, 454, 459-463</sup> The development of a strategy that allows the early detection of the majority of the resistant isolates, in the Lisbon Health Region context, is underway.

Another application concerning the comprehension of resistance-conferring mutations in a given population is its use as secondary epidemiologic markers in the discrimination of genetic clusters, as previously reported.<sup>405</sup> All clinical isolates in this study have been genotyped by MIRU-VNTR and all genetic clusters obtained were also analysed considering the mutations found. Several clusters contained isolates in which sequential acquisition of resistance could be observed, namely the acquisition of resistance to EMB or PZA and subsequent spreading in the community; resistance to PZA, shown the great mutational diversity in the same cluster, generally seems to be the last acquired resistance. A cluster's genetic pool diversification may result in a possible increase in the relative fitness of such strains; Lisboa3 cluster is the most represented cluster and the one that shows the highest diversity.

Comparing with previous studies involving the characterizations of strains from Lisbon Health Region, we have determined that Lisboa3 remains as the most predominant cluster, not only in Lisboa family, but also in the context of the Lisbon Health Region super family of isolates.<sup>26, 405</sup> For that reason, we conclude that strains belonging to this cluster have never been contained, continuing to cause more MDR-TB cases in the community, and at this stage, probably throughout the country.

We have verified that the observed clusters could be divided into smaller clusters composed by mutational identical isolates. The combination of the MIRU-VNTR genotyping with a

mutational analysis therefore allowed the discrimination of what would be considered an outbreak or a series of possible transmission events into minor ones. Recently, a new set of mycobacterial interspersed repetitive *loci* have been proposed for standardization of MIRU-VNTR technique, this new adjustment may significantly improve the technique discrimination power *per se*.<sup>56</sup>

Another aspect focused on this study was the prevalence of isolates with extensive drug resistance (XDR-TB) among the studied MDR-TB isolates, which was found to be 56%. Previous studies in other countries indicated XDR-TB (at the time still considered as MDR-TB resistance plus resistance to at least three classes of second-line drugs) prevalence of 10.9%, 19%, 15% and 4% among MDR-TB isolates in Iran, Latvia, South Korea and United States respectively.<sup>443, 464</sup> The prevalence of XDR-TB here obtained taking in account the former definition is 64%, which is significantly higher than any XDR-TB prevalence ever reported. This high level of XDR-TB strains is probably a result of a more than 10 years of MDR-TB strains circulating in Portugal.<sup>26</sup> These MDR-TB strains were first identified in the beginning of the 90s, as belonging to a particular family of strains genetically related, family Lisboa. The continuous dissemination of these MDR strains in the community has permitted the progressive acquisition of resistances to second-line drugs. Another issue that might be a contribution to the high prevalence of XDR-TB is related to the high frequency of FQs prescription in other infectious diseases. Portugal is the EU country with the highest rate of FQs prescription, about 3,10 DID (diary dose per 1000 inhabitants /day).<sup>465</sup> During the 1990s there was a gradual increase in the general use of quinolones for a systemic use, especially second-generation quinolones, and in the current millennium, third-generation quinolones for the treatment of respiratory tract infections. Excessive use of quinolones is inevitably associated with development of resistance and may contribute to selection of resistant *M. tuberculosis* strains.

In conclusion, MDR-TB cases in Lisbon Health region are due to *M. tuberculosis* strains belonging to a family of several clusters identified several years ago as already related to MDR-TB. The non-containment of such strains will continue to cause more MDR-TB cases as these strains spread through the local community, hospitals or even throughout the country. The development of a locally adapted and rapid molecular diagnosis method is necessary for the implementation of an effective MDR-TB fast-track plan. This will allow a more rapid detection and prevent a further increase of the already high, XDR-TB rate. The present study contributes not only with the knowledge of the present situation, but also with a first step towards the creation of such strategy.

## **2.6 ACKNOWLEDGEMENTS**

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# **Genetic Analysis of Extensively Drug-Resistant *Mycobacterium tuberculosis* strains in Lisbon, Portugal**

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### 3.1 SYNOPSIS

**Objectives:** XDR-TB threatens the global control of TB worldwide. Lisbon has a high XDR-TB rate (50% of the MDR-TB), which is mainly associated with Lisboa family strains. Few studies have addressed the identification of mutations associated with resistance to second-line injectable drugs and, the relative frequency of such mutations varies geographically. The aim of this study was to characterize the genetic changes associated with the high number of XDR-TB cases in Lisbon.

**Methods:** In the present study we have analyzed 26 XDR-TB clinical isolates. The *gyrA*, *tlyA* and *rrs* genes were screened for mutations that could be responsible for resistance to FQs and second-line injectable drugs. Moreover, the strains under analysis were also genotyped by MIRU-VNTR.

**Results:** The mutational analysis identified the most frequent mutations in the resistance-associated genes: S91P in *gyrA* (42.3%); A1401G in *rrs* (30.8%) and, Ins755GT in *tlyA* (42.3%). The occurrence of mutations in *rrs* was associated with the non-occurrence of mutations in *tlyA*. The genotypic analysis revealed that the strains were highly clonal, belonging to one of two MIRU-VNTR clusters, with the largest belonging to family Lisboa. Association between mutations in *gyrA* and *rrs* or *tlyA* was verified.

**Conclusions:** The association of specific mutations highlighted the strains' high clonality and indicates recent XDR-TB transmission. In addition, the identification of the most frequent resistance-associated mutations will be invaluable in applying XDR-TB molecular detection tests in the region in the near future.

### **3.2 INTRODUCTION**

XDR poses a serious threat to TB management. XDR-TB is defined as multidrug resistance MDR, *i.e.* resistance to at least INH and RIF, plus resistance to a FQ and any of the injectable second-line drugs (AMK, CAP or KAN).<sup>466</sup>

At least four different antimycobacterial drug classes are required for an adequate treatment and in the majority of the XDR-TB cases there are not enough options.<sup>467</sup> Resistance to FQs and second-line injectable drugs is indeed associated with poor outcomes.<sup>468</sup> As a consequence, such strains are not only very difficult to treat but also to eliminate.

In recent years, Portugal has had TB rates above the EU average, especially if we consider only the first 15 countries that have joined the EU (EU-15) and in which Portugal is included.<sup>469</sup>

Ten years ago, some of us described a family of strains causing a MDR-TB outbreak in the Lisbon Health Region. This family was later designated as the Lisboa Family.<sup>26</sup>

More recently, in one of our latest reports, we analysed a panel of multidrug-resistant isolates and observed that  $\approx 50\%$  were XDR-TB isolates; to our knowledge the highest XDR-TB rate ever reported.<sup>24</sup> However, the genetic basis of such resistance was unknown.

In this study, and given the paucity of studies characterizing such mutations in different settings, we aimed at determining which genetic changes are responsible for resistance to injectable second-line drugs and FQs and, association with specific clusters in Lisbon, Portugal.

### **3.3 METHODS**

#### **Clinical Isolates**

We have analyzed 26 XDR-TB clinical isolates, each corresponding to a different patient. These isolates were recovered from several hospital units and laboratories of Lisbon's Health Region during the year of 2005, and correspond to 74.3% of all the XDR isolated in this region, in this year. The remaining isolates were unviable and so were not processed. Our laboratory received 67 new and previously treated MDR-TB cases, 35 (52.2%) being XDR-TB.

**DST.** Isolates were tested for first-line drug susceptibility by the BACTEC 960 MGIT methodology according to the manufacturer's instructions. MDR-TB isolates were tested for second-line drug susceptibility by the radiometric BACTEC 460, as previously described.<sup>24</sup>

### PCR Amplification and DNA Sequencing

A 981 bp fragment, containing the *tlyA* gene, was amplified by PCR using *tlyAF3* (AAGGCATCGCACGTCGTCTTTCC) and *tlyAR3* (TGTCGCCCAATACTTTTCTACGC) primers; the region comprehended between the positions 1151 and 1590 of the *rrs* gene was amplified by PCR using primers RRS2-F (TGCCGGGGTCAACTCGGAGG) and RRS2-R (GAACCCCTCACGGCCTACGC); and, a 320 bp (78-397) internal fragment of the *gyrA* gene was amplified by PCR with oligonucleotide primers *gyrA-F* (CAGCTACATCGACTATGCGA) and *gyrA-R* (GGGCTTCGGTGCTACCTCAT). Cycling conditions were as follows: denaturation at 94°C for 4 minutes; 35 cycles of denaturation at 94°C for 1 min, annealing at 58°C (55°C for *gyrA*) for 1 min and extension at 72°C for 2 min and 30 sec (1 min for *gyrA*); and, lastly, a final extension step at 72°C for 10 min was performed. Crude DNA extracts of each isolate were used in all molecular biology reactions.

Prior to sequencing, the amplified fragments were purified using the MSB™ Spin PCRapace (Invitex™). The sequencing reactions were carried out with BigDye™ Terminator Cycle Sequencing Kit with AmpliTaq DNA polymerase (Applied Biosystems™) using primers *tlyAF1* (GTTGTTGGCCGCCCTGGAGT) and *tlyAR1* (GGTCTCGGTGGCTTCGTCGC), RRS2-R, or *gyrA-F* as appropriate.

All the mutations found were compared with those included in the Tuberculosis Drug Resistance Mutation Database ([www.tbdreamdb.com](http://www.tbdreamdb.com)).

### MIRU-VNTR

All isolates were also genotyped by the 12 *loci* Mycobacterial Interspersed Repetitive Unit – Variable Number of Tandem Repeats (MIRU-VNTR) as described previously.<sup>24</sup>

### Statistical Analysis

The statistical association between clusters and/or mutations was done using a two-tailed Fisher's Exact Test in GraphPad Prism™ Version 4.00 (GraphPad Software, Inc).

### 3.4 RESULTS

For this study we analysed 26 different XDR-TB isolates collected from Lisbon Health Region's hospital units during 2005. In order to determine the possible molecular basis for resistance to FQs and injectable second-line drugs, the isolates were screened for mutations in three genes – *gyrA*, *rrs* and *tlyA*. We preferentially screened the *gyrA* gene and not *gyrB* in this set of isolates since previous results on Lisbon FQ-resistant isolates indicates that *gyrB* mutations associated with resistance are absent in these strains (R. Macedo, unpublished results). We identified three different missense mutations in GyrA (D94A, D94G and S91P), the most common being mutation S91P which was present in 11 (42.3%) isolates (Table 3.1).

Concerning the mutations associated with resistance to injectable second-line drugs, all the analyzed isolates, except one, had mutations occurring in either *tlyA* or *rrs* genes. Occurrence of a mutation in *tlyA* gene was associated with the non-occurrence of a mutation in *rrs* gene (Fisher's Exact Test,  $p=0.0010$ ). The most prevalent mutations found in those genes were A1401G in *rrs* and a newly described frameshift mutation in *tlyA*, Ins755GT, which alone was present in 42.3% of the analysed isolates (Table 3.1).

When comparing genotypes, isolates were divided into two large MIRU-VNTR clusters (Table 3.2). One of the clusters belongs to the highly predominant Lisboa family and the other was previously designated by Q1 and presents a high genetic similarity with Lisboa strains. We verified that mutations occurring in *gyrA*, *rrs* and *tlyA* genes were cluster-associated. GyrA D94A and *rrs* A1401G occurred exclusively in cluster Q1, and GyrA D94G, S91P and *tlyA* Ins755GT were exclusive to the Lisboa3 cluster (Table 3.2). Different mutations at the same codon (GyrA94) were concordant with the MIRU-VNTR clustering analysis. Eleven of 17 FQ-resistant isolates on Lisboa3 cluster had the GyrA S91P mutation, which was strongly associated with *tlyA* Ins755GT mutation (Fisher's Exact Test,  $p<0.0001$ ). In cluster Q1, eight isolates had the GyrA D94A mutation, which was associated with *rrs* A1401G mutation (Fisher's Exact Test,  $p<0.0001$ ). Such isolates, in both clusters, may therefore illustrate several cases of primary XDR-TB transmission, although no patient data was available to establish epidemiological relatedness or provide evidence regarding the chain of transmission.

None of the mutations associated with resistance found in this study were detected in susceptible strains.

**Table 3.1** – Mutational frequency found on the resistance-associated genes of the 26 studied isolates.

Gene	Mutation (nt)	Mutation (aa)	No. of Isolates (%)
<i>gyrA</i>			
	T271C	S91P	11 (42.3)
	A281C	D94A	8 (30.8)
	A281G	D94G	6 (23.1)
	None	None	1 (3.8)
	Total		26 (100)
<i>rrs</i>			
	G1158T	-	1(3.8)
	A1401G	-	7(26.9)
	A1401G+G1158T	-	1(3.8)
	G1484T	-	1 (3.8)
	G1484T+C1402A	-	1 (3.8)
	Ins1211G	-	1 (3.8)
	Ins1512G	-	1 (3.8)
	None	-	13 (50.0)
	Total		26 (100)
<i>tlyA</i>			
	A205C	K69Q	1 (3.8)
	G223T	E75X	2 (7.7)
	Ins521CG	Frameshift	1 (3.8)
	Ins755GT	Frameshift	11 (42.3)
	None	None	11 (42.3)
	Total		26 (100)

**Table 3.2** – Resistance patterns, clusters and mutations found in the analysed isolates.

Isolate	First-Line		Mutations <sup>c</sup>					MIRU-VNTR
	Resistance Profile <sup>a</sup>	Second-Line Resistance Profile <sup>b</sup>	<i>inhA</i>	<i>rpoB</i> <sup>d</sup>	<i>gyrA</i>	<i>tlyA</i>	<i>rrs</i>	Cluster
BK519	IRSEP	CAP, KAN, CIP, OFX, ETH,	C-15T	C1349T (S531L)	A281G (D94G)	none	C1402A; G1484T	Lisboa3
BK128	IRSEP	CAP, AMK, KAN, CIP, OFX, ETH	C-15T	C1349T (S531L)	A281G (D94G)	G223T (E75X)	G1158T	Lisboa3
BK87	IRS	CAP, AMK, KAN, CIP, OFX, ETH	C-15T	C1349T (S531L)	A281G (D94G)	none	G1484T	Lisboa3
BK530	IRSEP	CAP, CIP, OFX, ETH	C-15T	C1349T (S531L)	T271C (S91P)	Ins755GT	Ins1211G	Lisboa3
BK872	IRSEP	CAP, KAN, CIP, OFX, ETH, PAS	C-15T	C1349T (S531L)	T271C (S91P)	Ins755GT	Ins1512G	Lisboa3
BK103	IRSEP	CAP, KAN, CIP, OFX, ETH	C-15T	C1349T (S531L)	A281G (D94G)	A205C (K69Q)	none	Lisboa3
BK232	IRSEP	CAP, AMK, KAN, CIP, OFX, ETH, PAS, CS	C-15T	C1349T (S531L)	A281G (D94G)	G223T (E75X)	none	Lisboa3
BK643	IRSEP	CAP, KAN, CIP, OFX, ETH	C-15T	C1349T (S531L)	A281G (D94G)	Ins521CG	none	Lisboa3
BK30	IRSEP	CAP, KAN, CIP, OFX, ETH	C-15T	C1349T (S531L)	T271C (S91P)	Ins755GT	none	Lisboa3
BK81	IRSEP	CAP, KAN, CIP, OFX, ETH	C-15T	C1349T (S531L)	T271C (S91P)	Ins755GT	none	Lisboa3
BK127	IRSEP	CAP, AMK, KAN, CIP, OFX, ETH, CS	C-15T	C1349T (S531L)	T271C (S91P)	Ins755GT	none	Lisboa3
BK214	IRSEP	CAP, AMK, KAN, CIP, OFX, ETH, PAS, CS	C-15T	C1349T (S531L)	T271C (S91P)	Ins755GT	none	Lisboa3
BK309	IRSEP	CAP, KAN, CIP, OFX, ETH, PAS	C-15T	C1349T (S531L)	T271C (S91P)	Ins755GT	none	Lisboa3
BK548	IRS	CAP, KAN, CIP, OFX, ETH, PAS	C-15T	C1349T (S531L)	T271C (S91P)	Ins755GT	none	Lisboa3
BK801	IRSE	CAP, CIP, OFX, ETH	C-15T	C1349T (S531L)	T271C (S91P)	Ins755GT	none	Lisboa3
BK839	IRSEP	CAP, KAN, CIP, OFX, ETH, PAS	C-15T	C1349T (S531L)	T271C (S91P)	Ins755GT	none	Lisboa3
BK1144	IRSEP	CAP, KAN, CIP, OFX, ETH, PAS	C-15T	C1349T (S531L)	T271C (S91P)	Ins755GT	none	Lisboa3
BK104	IREP	CAP, AMK, KAN, CIP, OFX, ETH	C-15T	C1349T (S531L)	A281C (D94A)	none	A1401G	Q1
BK269	IREP	CAP, AMK, KAN, CIP, OFX, ETH	C-15T	A1304T (D516V)	A281C (D94A)	none	A1401G	Q1
BK364	IRSEP	CAP, AMK, KAN, CIP, OFX, ETH, PAS, CS	C-15T	C1349T (S531L)	A281C (D94A)	none	A1401G	Q1
BK460	IREP	CAP, AMK, KAN, CIP, OFX, ETH	C-15T	C1349T (S531L)	A281C (D94A)	none	A1401G	Q1
BK474	IRSEP	CAP, AMK, KAN, CIP, OFX, ETH	C-15T	C1349T (S531L)	A281C (D94A)	none	A1401G	Q1
BK667	IREP	CAP, AMK, KAN, CIP, OFX, ETH, PAS	C-15T	C1349T (S531L)	A281C (D94A)	none	A1401G	Q1
BK733	IREP	CAP, AMK, KAN, CIP, OFX, ETH	C-15T	C1349T (S531L)	A281C (D94A)	none	A1401G	Q1
BK396	IRSEP	CAP, AMK, KAN, CIP, OFX, ETH,	C-15T	C1349T (S531L)	A281C (D94A)	none	A1401G; G1158T	Q1
BK372	IR	CAP, AMK, KAN, CIP, OFX, ETH,	C-15T	C1349T (S531L)	none	none	none	Q1

<sup>a</sup> I – isoniazid; R – rifampicin; E – ethambutol; S – streptomycin; P – pyrazinamide.



<sup>b</sup> CAP – capreomycin; AMK – amikacin; KAN – kanamycin; CIP – ciprofloxacin; OFX – ofloxacin; ETH – ethionamide; PAS – para-amino salicylic acid; CS – cycloserine.

<sup>c</sup> Nucleotide mutation with resulting amino acid mutation in brackets, if applicable.

<sup>d</sup> nucleotidic mutational position relative to *M. tuberculosis* H37Rv *rpoB*; aminoacidic positioning is relative to *E. coli* numbering.

### 3.5 DISCUSSION

We have examined the main genes associated with resistance to FQs and injectable second-line drugs – *gyrA*, *rrs* and *tlyA*, respectively. The mutations found in the *gyrA* gene have been previously described as associated with FQ resistance, and our results support this.

In contrast, the screening of *rrs* and *tlyA* yielded mutations not previously described. Twenty-five isolates displayed mutations in one of these genes and only three isolates had simultaneous mutations in both genes. Our results also indicate that only a subset of the mutations conferring KAN-resistance actually confers resistance to AMK.<sup>359, 470</sup> The data seem to support the perception that resistance to AMK is associated with A1401G mutations but not with *tlyA* mutations, while nine KAN-resistant isolates exhibited *tlyA* mutations only. However, in a study by Maus *et al* nonsense *tlyA* mutants remained susceptible to KAN and AMK, which indicates that *tlyA* mutations probably have no role in resistance to these second-line aminoglycosides.<sup>375</sup> We cannot, however, exclude the existence of mutations outside the analyzed *loci*, since mutants resistant to KAN with no *tlyA* or *rrs* mutations have been found. In addition, our results support previous findings in that mutations in *rrs* can mediate cross-resistance between CAP, KAN and AMK.<sup>359</sup> On the other hand, our results clearly differ from the results obtained by Jugheli *et al* who have found no mutations in the *tlyA* gene of 145 isolates of which 78 and 66 were KAN- and CAP-resistant, respectively.<sup>470</sup>

A new reverse hybridization based test to screen for cultured XDR-TB isolates - GenoType MTBDRs/ (Hain Lifescience, GmbH, Nehren, Germany)<sup>471</sup> is already available commercially. This new test detects mutations associated with resistance to second-line injectable drugs and FQs. According to results obtained in our study, this test would have been able to detect 96.2% of the FQ resistance and 34.6% of the resistance to CAP, KAN or AMK. Since resistance-associated mutations vary geographically we therefore stress the need for knowledge concerning the prevalence of such mutations before *in situ* implementation of these commercial assays.

Another important conclusion concerns the clonality of the analysed strains. These strains were shown to be highly clonal, belonging to one of two close genetic clusters. Also important,

was the evidence that a large proportion of the analysed cases (73.1%) were possibly primary XDR-TB. This shows that the appropriate means to avoid transmission are not in place since active transmission of these deadly forms of TB is taking place.

The strains analysed in this study are highly resistant, presenting clinicians with few options. Four to six drugs would be necessary to treat these patients and only 4 of the 26 isolates in this study appear to be susceptible to at least four of these drugs. In the remaining isolates, the so-called third-line drugs (CFZ, linezolid, amoxicillin/clavulanate, imipenem, macrolides), which have *in vitro* activity but with limited clinical data, should be explored.<sup>467</sup>

The present study has, consequently, important implications for the treatment, diagnosis and epidemiology of XDR-TB in the Portuguese setting. Also, it contributes to the global awareness of XDR/MDR-TB, and the urgency for new therapeutic options and novel molecular diagnosis tests adapted to specific settings. This study also highlights the utmost importance of strict regulation in the prescription of antibacillary drugs, without which is impossible to effectively control and eliminate TB, even with new antibacillary drugs.

### **3.6 ACKNOWLEDGEMENTS**

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### **3.7 FUNDING**

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### **3.8 TRANSPARENCY DECLARATIONS**

None to declare



# **Tuberculosis Drug-Resistance in Lisbon, Portugal: a 6-Year Overview**

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## 4.1 ABSTRACT

MDR and extensive XDR pose a serious threat to TB management in Portugal. The country has high TB incidence rates in comparison with the other European Union countries, with Lisbon Health Region being one of the most affected regions.

In the present study we have analyzed a convenience sample of 3 025 *M. tuberculosis* clinical isolates, recovered over a six-year period (2001-2006) in Lisbon Health Region, regarding drug-resistance to both first-line and second-line drugs. Moreover, one hundred of these isolates were also genotyped by 12-*loci* Mycobacterial Interspersed Repetitive Unit – Variable Number of Tandem Repeats (MIRU-VNTR) analysis.

We have compared each year and observed the existence of 22 different resistance profiles with MDR-TB rates ranging between 9.9% and 15.2% and XDR-TB rates, relative to the number of MDR-TB isolates, between 44.3% and 66.1% (excluding one year here considered as an outlier). A steady increase in the fraction of MDR-TB isolates resistant to all first-line drugs was also noticed. The genotyping analysis of MDR-TB isolates revealed six clusters, of which three (Lisboa3, Lisboa4 and Q1) were related with XDR-TB.

Our results show that active transmission of MDR- and XDR-TB is taking place and that the high prevalence of observed XDR-TB is due to the continued transmission of particular genetic clusters. An early detection of resistant cases would greatly benefit from the enforcement in case detection and implementation of genotyping in diagnostic routines.

## **4.2 INTRODUCTION**

The worldwide control of TB infection and transmission may be severely hampered by the recurrent development of resistance to antibacillary drugs. MDR and XDR constitute a special and more serious case of resistance because it renders ineffective the most efficient first- and second-line drug therapies, respectively.

Although TB cases resistant to second-line drugs were widely known, the growing awareness concerning the widespread and growing rates in industrialized nations highlighted the necessity to recognize and classify this old problem.<sup>443</sup> According to the latest WHO report, at least one XDR-TB case has been described in 58 countries.<sup>472</sup> Other reports also highlight XDR-TB as a very serious global public health crisis. The survival rates associated with XDR-TB are low compared with MDR-TB or susceptible TB. Population studies performed in Russia, South Korea and Peru indicated survival rates of 48%, 54% and 60%, respectively among HIV-negative patients.<sup>473-475</sup>

The tuberculosis situation in Portugal is worrisome. The incidence rate is high, taking in account the incidence rates of the other European Union members and the recent reports by some of us concerning the high prevalence of MDR-TB and XDR-TB in the Lisbon Health Region.

Genotyping of clinical isolates would allow a better identification of transmission chains. The main genetic clusters present in the region were already identified and, the majority described more than ten years ago. Such isolates apparently continue to circulate in the region and have inclusively spread to other regions in the country without noticeable limitations.<sup>24, 26</sup>

The INSA-RJ laboratory has, over the years, collected and analyzed a high number of TB clinical isolates, mainly from Lisbon Health Region. In the present report, we analyze the prevalence of resistant isolates, including MDR- and XDR-TB, over a six-year period (2001-2006).

## **4.3 METHODS**

### **Clinical Isolates**

We have analyzed the data records of all clinical isolates sent for identification and drug susceptibility tests at the INSA-RJ, Portugal's national TB Reference Laboratory. For the entire study, a convenience sample of 3 025 isolates were selected from our tuberculosis database.



The selection of these isolates was based on: complete information concerning drug susceptibility testing; isolation in Lisbon Health Region. Isolates with no *Mycobacterium tuberculosis* complex identification were discarded and, duplicate isolates were removed from the data set. These criteria allowed the selection of 283 isolates from 2001, 741 from 2002, 667 from 2003, 547 from 2004, 442 from 2005 and 345 from 2006.

## DST

All isolates were tested for susceptibility to first-line drugs (INH, RIF, STP, EMB and PZA) by the BACTEC 960™ MGIT™ or BACTEC 460™ (Becton-Dickinson™) method according to the manufacturer's indications. Isolates resistant to INH and RIF were considered MDR-TB and tested for second-line drug susceptibility.

Second-line DST was performed by the BACTEC 460™ system to CAP (1,25 mg/L), AMK (1,0 mg/L), KAN (5,0 mg/L), CIP (1,0 mg/L), OFX (2,0 mg/L), ETH (5,0 mg/L) and PAS (4,0 mg/L), according to Pfyffer *et al.*<sup>446</sup> MDR-TB isolates resistant to, at least, a FQ (OFX or CIP) and a second-line injectable drug (CAP, KAN or AMK) were considered XDR-TB isolates.

## MIRU-VNTR Genotyping

One hundred isolates (28 from 2004, 48 from 2005 and, 24 from 2006) were genotyped by the automated 12-*loci* MIRU-VNTR technique as described by Supply *et al.*<sup>57</sup>

## 4.4 RESULTS

For this study, the records of a total of 3 025 clinical isolates were analysed. The analysis of these isolates allows us to assess the TB situation regarding drug resistance among all isolates tested at the TB National Reference Laboratory from Portugal's National Institute of Health Dr. Ricardo Jorge. This convenience sample may also provide a general picture of the prevalence of MDR-TB and XDR-TB in Lisbon Health Region.

We started by analyzing the isolates resistant to first-line antibacillary drugs. Overall, we found 22 different resistant profiles in isolates resistant to one or more first-line drugs distributed through the years (2001-2006). Eight profiles met the criteria for MDR-TB, with 2003 registering the highest number of MDR-TB cases (82) (Table 4.1).

**Table 4.1** – First-line resistant profiles found among the analyzed isolates in each year.

Resistance Profile <sup>a</sup>	No. of clinical isolates studied per year					
	2001	2002	2003	2004	2005	2006
Pan-susceptible	220	548	503	424	331	268
E	0	0	0	0	0	2
ES	0	0	0	0	0	1
I	4	26	6	12	14	5
ISE	0	0	0	0	1	0
ISEP	0	0	0	2	0	0
IP	2	2	1	2	1	1
IR	2	10	3	0	3	3
IRE	0	0	0	0	1	1
IREP	3	4	0	2	9	3
IRSE	4	2	6	4	2	0
IRSEP	11	21	32	24	30	18
IRP	1	7	7	4	4	1
IRS	7	6	7	4	5	1
IRSP	12	31	27	19	13	7
IS	5	13	16	16	9	10
ISP	0	2	1	0	1	0
P	0	0	0	0	5	0
R	0	3	2	3	0	1
RP	0	0	1	0	0	0
RS	0	1	0	0	0	0
S	12	65	54	31	12	23
SP	0	0	1	0	1	0
<b>Total</b>	<b>283</b>	<b>741</b>	<b>667</b>	<b>547</b>	<b>442</b>	<b>345</b>

<sup>a</sup> - I – isoniazid, R – rifampicin, S – streptomycin, E – ethambutol and P – pyrazinamide.

The analysis of the susceptibility to second-line drugs yielded 26 different resistant patterns, 15 of which corresponded to XDR-TB isolates (Table 4.2). From 2001 to 2006, 14 (2006) to 47 (2003) XDR-TB cases were detected in this study.

**Table 4.2** – Second-line resistant profiles found among the analyzed isolates in each year.

Resistance Profiles <sup>a</sup>	No. of clinical isolates studied per year					
	2001	2002	2003	2004	2005	2006
Pan-susceptible <sup>a</sup>	2	6	7	7	12	3
CAP AMK CIP OFX ETH	0	0	0	1	0	1
CAP AMK ETH PAS	0	0	1	0	0	0
CAP AMK KAN CIP ETH	1	1	0	0	0	0
CAP AMK KAN CIP ETH PAS	0	3	0	0	0	0
CAP AMK KAN CIP OFX ETH	4	5	10	14	11	3
CAP AMK KAN CIP OFX ETH PAS	0	0	1	5	7	2
CAP AMK KAN CIP OFX PAS	0	0	0	0	1	1
CAP AMK KAN ETH	0	1	4	1	2	0
CAP AMK KAN ETH PAS	0	0	1	0	0	0
CAP AMK KAN OFX	0	0	1	0	0	0
CAP CIP ETH	1	0	1	0	0	0
CAP CIP ETH PAS	0	1	0	0	0	0
CAP CIP OFX ETH	2	3	3	1	1	0
CAP ETH	0	0	0	0	1	0
CAP ETH PAS	0	0	0	0	1	0
CAP KAN CIP OFX ETH	4	6	20	8	7	3
CAP KAN CIP OFX ETH PAS	0	5	7	1	6	2
CAP OFX ETH	0	0	0	1	0	0
CIP ETH	0	2	1	1	1	0
CIP OFX	0	1	0	1	0	0
CIP OFX ETH	5	4	5	2	4	1
CIP OFX ETH PAS	0	4	1	0	0	0
ETH	9	35	17	10	17	2
KAN CIP OFX ETH	6	6	4	5	2	1
KAN CIP OFX ETH PAS	0	5	1	1	0	1
ND	249	653	581	488	369	325
OFX	0	0	1	0	0	0
<b>Total</b>	<b>283</b>	<b>741</b>	<b>667</b>	<b>547</b>	<b>442</b>	<b>345</b>

<sup>a</sup> CAP – capreomycin; AMK – amikacin; KAN – kanamycin; CIP – ciprofloxacin; OFX – ofloxacin; ETH- ethionamide; PAS – para-amino salicylic acid; ND – not determined.

The trend in the MDR-TB and XDR-TB cannot be clearly evaluated since the varying number of clinical samples received by the laboratory for testing may act as a confounding factor in such analysis. Nevertheless, considering the years of 2002-2005, an increase in the MDR-TB may have occurred because in 2005 the MDR-TB cases accounted for 15.3%, an increase of approximately 4% considering the three previous years (Table 4.3). Also, the percentage of MDR-TB isolates with resistance to all first-line drugs appears to show a steady increase from 2001/2002 to 2006 varying between 25.9% in 2002 and 52.9% in 2006. Considering the XDR-TB rate, and taking into account the year of 2006 as an outlier given the low number of tested strains, we verify that it varied between 44.3% (2002) and 66.1% (2004) with a mean value of 54.3%.

Twelve-*loci* MIRU-VNTR genotyping analysis was implemented in 2004, and for that reason it was not possible to assess the genetic clusters circulating in the previous years. Genotyping data was available for one hundred of the selected MDR-TB isolates from 2004-2006. A MIRU-VNTR dendrogram of the genotyped isolates was produced for each year. Genetic clusters were defined as a group of two or more isolates exhibiting the same MIRU-VNTR pattern. A clear predominance of Lisboa family strains was verified between 2004 and 2006. Six clusters were identified, with three belonging to Lisboa family (Lisboa3, Lisboa4 and Lisboa10) (Figure 4.1). Lisboa3, Lisboa 4 and Q1 clusters were associated with XDR-TB, with Lisboa3 having the highest number of XDR-TB cases (Table 4.4).

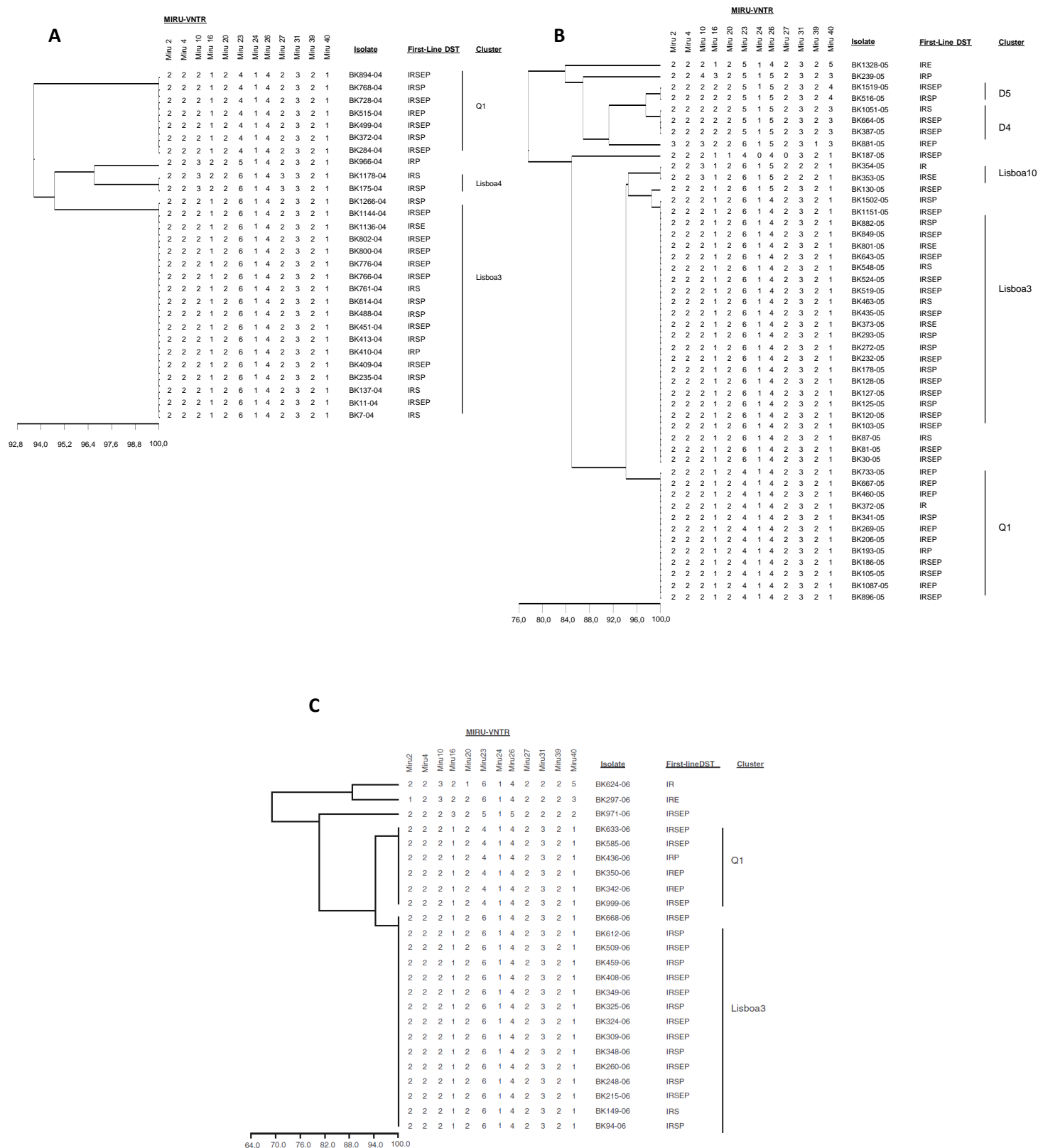
**Table 4.3** – Number and percentage of resistant, MDR- and XDR-TB isolates, distributed by each year (2001-2006).

Resistance	No. of isolates (%)					
	2001	2002	2003	2004	2005	2006
Total	283	741	667	547	442	345
Resistant <sup>a</sup>	63 (22.3)	193 (26.0)	164 (24.6)	123 (22.5)	111 (25.1)	77 (22.3)
MDR-TB <sup>b</sup>	40 (14.1)	81 (10.9)	82 (12.3)	57 (10.4)	67 (15.3)	34 (9.9)
XDR-TB <sup>c</sup>	18 (52.9)	35 (44.3)	47 (57.3)	37 (66.1)	34 (50.7)	14 (73.6)

a – Isolates resistant to one or more antibacillary drugs;

b – Isolates resistant to at least INH and RIF;

c – XDR-TB rate expressed as the fraction of the tested MDR-TB isolates with resistance to at least one second-line injectable drug and a FQ.



**Figure 4.1** – MIRU-VNTR dendrograms of MDR-TB isolates recovered in the years of 2004 (A), 2005 (B) and, 2006 (C). First-Line DST: I – isoniazid, R – rifampicin, S – streptomycin, E – ethambutol and P – pyrazinamide.

**Table 4.4** – Distribution of the MDR-TB genotyped isolates *per cluster per year* and, number of XDR-TB isolates in each cluster.

MIRU-VNTR Cluster	2004		2005		2006	
	No. of	No. of XDR	No. of	No. of XDR	No. of	No. of XDR
	MDR	Isolates	MDR	Isolates	MDR	Isolates
	Isolates		Isolates		Isolates	
D5	-	-	2	0	-	-
D4	-	-	3	0	-	-
Lisboa3	18	11	24	18	15	13
Lisboa4	2	1	-	-	-	-
Lisboa10	-	-	2	0	-	-
Q1	7	3	12	8	6	3

- Not detected in that year

#### 4.5 DISCUSSION

Portugal is a country with a considerable TB incidence taking in account the other EU countries. The high number of XDR-TB cases and rates in an area of 14 572 km<sup>2</sup> (Lisbon, Setúbal and Santarém districts) are a concern to those who have to manage this problematic situation.

In a previous publication we have pointed to two possible explanations for the high prevalence of XDR-TB in Portugal: the non-containment of Lisboa Family strains and the high FQ prescription rate.<sup>24, 465, 476</sup> Additionally, the use of the WHO-recommended standard treatment regimen for MDR-TB suspected cases with unavailable first-line drug susceptibility tests results may potentiate this situation.<sup>203</sup> This treatment regimen includes a six month initial phase with KAN, ETH, a FQ, PZA and EMB. In a previous study, we have shown that 92% of the MDR-TB isolates were also resistant to ETH, mostly due to cross-resistance between INH and ETH and, that 79.4% of the same MDR-TB isolates were also resistant to EMB and/or PZA.<sup>24</sup> In the present study, that proportion ranged from 77.5 to 93.0% and resistance to ETH among all tested isolates was seen to range from 80.0 to 94.1%. Consequently, the use of the MDR-TB standard treatment regimen in the Portuguese setting does not provide the patient with the necessary active drugs and, may lead to resistance acquisition to second-line injectable drugs and/or FQs and hence, to XDR-TB development.

The results presented here show that, in accordance with previous studies by some of us, XDR-TB is not only a current problem that must necessarily be addressed, but is the result of a continued circulation of MDR-TB clones detected in the 1990s.<sup>26</sup> The main clusters were detected and shown to maintain their prevalence, over the three year-period (where genotyping was available) in the MDR-TB/XDR-TB scenario of Lisbon Health Region.

The largest cluster of XDR-TB in the world, in South Africa, belongs to the KZN family of TB strains, described in 1996 and at the time pan-susceptible or resistant to first-line drugs only.<sup>477</sup> In Portugal, the situation is similar, Lisboa family, which was identified in 1996, was already strongly associated with MDR-TB at that period and now is associated with XDR-TB.<sup>24, 26, 478</sup> Monitoring of these strains through timely genotypic analysis would provide a mean to identify more rapidly MDR-TB and XDR-TB cases and ensure greater tracing of contacts, with the aim of containing these strains, thereby avoiding a possible XDR-TB outbreak. Given the high clonality that was found associated with MDR-TB and XDR-TB, and because genotyping results can be obtained earlier than DST results, the identification of an isolate belonging to a MDR-TB or XDR-TB associated cluster might be useful in identifying a possible MDR-TB or XDR-TB strain at an earlier stage.

Transmission can also be enhanced by another factor, the sputum smear-positive case rate. Sputum smear-positivity is associated with higher transmission and Portugal has a high sputum smear-positive case rate when compared with the majority of the EU countries, which *per se* may be predictive of a worse outcome.<sup>469, 479, 480</sup> The explanation to higher bacillary loads might be related with the strain itself or may be due to a delayed case detection. If effective measures are not put in place to avoid the transmission of these strains, it is conceivable that an increase of the inherently high treatment costs of MDR-TB and XDR-TB for national tuberculosis control programs may occur, diverting resources necessary to control TB.<sup>481</sup>

A limitation to this study is the fact that a convenience sample was used. Nevertheless, the high number of cases found within such reduced geographic region justifies particular attention and urgent implementation of control measures.

A study concerning XDR-TB treatment in Tomsk, Russia by Keshavjee *et al* showed that although there was a less favorable outcome it is possible to treat XDR-TB with regimens including five active drugs plus a FQ and CAP even when resistance was found.<sup>473</sup> In the Portuguese setting, as we have shown, given the higher degree of resistance, the problem resides in finding at least four active drugs. This study is an important contribution for the

knowledge concerning drug resistance in Portugal and improvement of standard treatment regimens.

#### **4.6 ACKNOWLEDGEMENTS**

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#### **4.7 TRANSPARENCY DECLARATIONS**

The authors declare that they have no conflicts of interest in relation to this work.



# **From Multidrug-Resistant Tuberculosis to Extensively Drug Resistance in Lisbon, Portugal: the stepwise mode of resistance acquisition**

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## 5.1 SYNOPSIS

The development and transmission of XDR-TB constitutes a serious threat to the effective control of tuberculosis in several countries. Resistance is the result of adaptive selection of *M. tuberculosis* strains that acquire and accumulate specific mutations at specific genes. Current mechanisms of drug resistance include the modification or overexpression of drug targets, drug efflux, inactivation of activator enzymes and overexpression of drug-modifying enzymes.

In the present study a set of 44 MDR or XDR-TB isolates were genotyped and screened for mutations in genes associated to second-line drug resistance, namely *tlyA*, *gyrA*, *rrs* and *eis* genes. We have determined the most prevalent mutations found in each gene to be Ins755GT in *tlyA*, A1401G in *rrs*, G-10A in *eis* and S91P in *gyrA*. Additionally, two genetic clusters were found in this study: Lisboa3 and Q1. The characteristic mutational profile found among recent XDR-TB circulating in Lisbon was also found in MDR-TB strains isolated in the 90s, highlighting the ancient XDR-TB problem in this region. Also investigated was the resistance level conferred by *eis* G-10A mutations, revealing that *eis* G-10A mutations may result in undetectable AMK resistance.

In conclusion, the analysis of the distribution of the mutations found by genetic cluster showed that in Q1 cluster two mutations, *gyrA* D94A and *rrs* A1401G, were enough to ensure development of XDR-TB from a MDR strain. Moreover, in Lisboa3 cluster it was possible to elaborate a model in which the development of KAN low-level resistance was at the origin of the emergence of XDR-TB strains that can be discriminated by *tlyA* mutations.

## 5.2 INTRODUCTION

XDR-TB is a consequence of multiple acquisitions of mutations in specific resistance-associated genes. Such mutations may interfere with drug-target interaction or, overexpress the protein target or other drug modification enzymes. Since XDR-TB is defined as resistance to INH and RIF plus resistance to a FQ and a second-line injectable antibacillary drug - CAP, AMK or KAN, the most frequently implicated genes are: *inhA*, *katG*, *rpoB*, *gyrA*, *rrs*, *tlyA* and *eis*.<sup>359, 375, 432</sup>

According to the latest drug resistance surveillance report from the WHO, XDR-TB cases have already been reported in over 58 countries. The worldwide occurrence of XDR-TB raises serious public health concerns as this level of cumulative resistance to the most effective and available antibacillary drugs is most often found to be untreatable.<sup>472</sup>

In Portugal, the high XDR-TB rates in its capital city Lisbon, also the region with the highest TB incidence, have shown to be mainly associated with particular genetic clusters that have undergone extensive clonal expansion in the community. Of special importance are the strains belonging to some of the Lisboa family genetic clusters that are in circulation for almost two decades. XDR-TB in the region has been considered a serious public health concern in the years following the first definition of XDR-TB, but recent studies now show that this is an old problem that can be, at least, tracked back to the 90s.<sup>26, 482</sup>

The main genetic clusters associated with MDR and XDR-TB in Lisbon, designated Lisboa3 and Q1, have been described in previous publications. Lisboa3 cluster was originally described in the 90s, highly associated with the original MDR-TB outbreak in the region, and presently with XDR-TB. The Q1 cluster, detected only in strains recovered from 2003, is genetically very close to the original Lisboa family and contains XDR-TB strains, although presenting a lesser mutational diversity when compared with Lisboa3 cluster.<sup>24-26, 405</sup> The exact phylogenetic origin of this later cluster in respect to Lisboa family is still under study as there are significant genetic differences regarding to other genomic *loci* or spoligotyping profiles (unpublished data).

Previous data from our laboratory has contributed to elucidate the molecular basis of resistance development among MDR-TB strains and XDR-TB in the region through the mutational analysis of *gyrA*, *rrs* and *tlyA*.<sup>25</sup> However, the mode of resistance acquisition and the genetic determinants leading to second-line aminoglycoside resistance in the region are not fully understood. Combining both mutational and genotypic approaches will eventually

lead to insights on how resistance develops and allows further strain discrimination for the study of transmission in the community.<sup>405</sup>

Zaunbrecher *et al*, associated promoter mutations in *eis* with KAN resistance. The gene product of *eis*, an acetyl-transferase, catalyzes multiacetylation of aminoglycosides, modifying KAN and, with lesser affinity, AMK. The explanation offered by the authors suggested that *eis* promoter mutations are responsible for KAN resistant isolates that do not present cross-resistance to AMK.<sup>371, 372</sup>

Here, in attempt to further elucidate the dynamics of resistance acquisition and investigate an eventual role for *eis* promoter mutations in second-line aminoglycoside resistance we have studied a set of MDR/XDR-TB isolates that included some of the original strains involved in the Lisbon MDR-TB outbreak from the 90s.<sup>26</sup>

### 5.3 METHODS

#### Clinical Isolates, Drug Susceptibility Testing and Genotyping

A total of 44 MDR/XDR *M. tuberculosis* clinical isolates recovered from TB patients of the Lisbon region, between 1997 and 2009, were studied. All strains were isolated at local hospital units and laboratories and sent for drug-susceptibility testing at Level 3 Mycobacteriology Laboratories. For strains isolated in 1997-1998 and in 2005, only DNA was available for mutational analysis and genotyping.

At the time of isolation, first-line DST for isolates recovered between 1997-98 was performed using the radiometric BACTEC™ 460 system (Becton Dickinson Diagnostic Systems, Sparks, MD, USA) for INH, RIF, EMB and STP. For strains isolated after 2005, first-line DST for the remaining isolates was carried out by the fluorimetric BACTEC™ MGIT™ 960 system (Becton Dickinson Diagnostic Systems, Sparks, MD, USA) for INH, RIF, EMB, STP, and PZA.

For isolates recovered in the year of 2005, DST to CAP, KAN, AMK and OFX was performed by the radiometric method BACTEC 460™, using the following critical concentrations: 1.25, 5.0, 1.0 and 2.0 mg/L, respectively. For isolates after 2005, DST to CAP, AMK and OFX was performed by the BACTEC™ MGIT™ 960 system using the recommended critical concentrations for second line drugs.<sup>483</sup>

All strains were genotyped by 12-*loci* MIRU-VNTR as described by Supply *et al* as it was the standard genotyping technique implemented in the laboratory during the years of 2005-2007.<sup>57</sup>

### Mutational analysis of drug-resistance associated genes

A 981 bp fragment, containing *tlyA* gene, was amplified by PCR using *tlyAF3* (5'-AAGGCATCGCACGTCGTCTTTCC-3') and *tlyAR3* (5'-TGTCGCCCCAATACTTTTCTACGC-3') primers. Cycling conditions were as follows: denaturation at 94°C for 4 min; 35 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min and extension at 72°C for 2 min and 30 sec; lastly, a final extension step at 72°C for 10 min was performed.

The region comprehended between the positions 1151-1590 of the *rrs* gene was amplified by PCR using primers *RRS2-F* (5'-TGCCGGGGTCAACTCGGAGG-3') and *RRS2-R* (5'-GAACCCCTCACGGCCTACGC-3'). Cycling conditions were as follows: denaturation at 94°C for 4 min; 35 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min and extension at 72°C for 2 min and 30 sec; lastly, a final extension step at 72°C for 10 min was performed.

A 601 bp fragment, containing the promoter region of *eis* gene, was amplified from all isolates by PCR using *eisF1* (5'-GCCATGGGACCGGTACTTGC-3') and *eisR1* (5'-GTAGATGCCGCCCTCGCTAG-3') oligonucleotide primers. Cycling conditions were as follows: denaturation at 94°C for 5 minutes; 35 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min and extension at 72°C for 1 min and 30 sec; and a final extension step at 72°C for 10 min.

An internal 320bp (78-397) fragment of the *gyrA* gene was amplified by PCR with oligonucleotide primers *gyrA-F* (5'-CAGCTACATCGACTATGCGA-3') and *gyrA-R* (5'-GGGCTTCGGTGACCTCAT-3'). Cycling conditions were as follows: denaturation at 94°C for 4 min; 35 cycles of denaturation at 94°C for 1 min, annealing at 58°C (55°C for *gyrA*) for 1 min and extension at 72°C for 2 min and 30s (1 min for *gyrA*); lastly, a final extension step at 72°C for 10 min was performed. Crude DNA extracts of each isolate were used in all molecular biology reactions. Prior to sequencing, the amplified fragments were purified using the MSB™ Spin PCRapace (Invitex™). Sequencing reactions and capillary electrophoresis were outsourced to MacroGen Inc using primers *eisF1*, *RRS-2R*, *gyrA-F*, *tlyAF1* (5'-GTTGTTGGCCGCCCTGGAGT-3') and *tlyAR1* (5'-GGTCTCGGTGGCTTCGTCGC-3'), as appropriate.

### AMK/KAN Minimal Inhibitory Concentration

AMK and KAN minimal inhibitory concentration (MIC) for selected isolates was determined using the Resazurin Microplate Assay (REMA) system as described by Martin *et al.*<sup>484</sup> Selected isolates were tested in duplicate for growth at serial two-fold dilutions of both antibacillary drugs at a concentration range between 80 and 0.08 mg/L. Growth controls with no drug and a sterility control were included in each plate assay.<sup>485</sup>

## 5.4 RESULTS

### Mutations in second-line drug resistance associated genes

In the present study, 44 MDR/XDR-TB strains were analyzed, 5 of which were isolated during the MDR-TB outbreak identified in the 90s. For all isolates, we have analyzed four genes associated with XDR criteria of resistance, injectable second-line drugs and FQs - *rrs*, *tlyA*, *eis*, and *gyrA*.

In *rrs* gene, six different mutations were found, including two insertions (Table 5.1). The most prevalent mutation found in *rrs* gene was A1401G (13/40), a classical mutation associated with KAN, AMK and CAP resistance, which was highly associated with the the Q1 cluster as previously reported.<sup>25</sup> This mutation was also found in one Lisboa3 isolate from 1998.

The mutational analysis of *tlyA* has revealed four different mutations: one missense mutation, one nonsense mutation and two frameshift mutations. All mutations detected in *tlyA* gene were detected in Lisboa3 cluster only, the most prevalent being a frameshift GT insertion at position 755 found in 13 isolates.

The screening of *eis* promoter region, more recently shown to be associated with KAN resistance, resulted in the finding of one mutation only, G-10A, previously shown by Zaunbrecher *et al* to cause overexpression of the *eis* gene.<sup>372</sup> This mutation was found in Lisboa3 isolates (19/27).

**Table 5.1-** Resistance patterns, clusters and mutations found in the 44 analyzed isolates.

Isolate	Year	First-Line	Second-Line					Mutations <sup>c</sup>			MIRU-VNTR
		Resistance	Resistance <sup>b</sup>								Cluster
		Profile <sup>a</sup>	KAN	AMK	CAP	OFX	<i>eis</i>	<i>tlyA</i>	<i>rrs</i>	<i>gyrA</i>	
BK103	2005	IRSEP	R	S	R	R	G-10A	A205C (K69Q)	none	A281G (D94G)	Lisboa3
BK232	2005	IRSEP	R	R	R	R	G-10A	G223T (E75X)	none	A281G (D94G)	Lisboa3
BK643	2005	IRSEP	R	S	R	R	G-10A	Ins521CG	none	A281G (D94G)	Lisboa3
FF359	1998	IRS	nd	nd	nd	nd	G-10A	none	A1401G	A281G (D94G)	Lisboa3
HPV129	2009	IRSEP	S	S	S	R	G-10A	none	none	A281G (D94G)	Lisboa3
HPV108	2009	IRSEP	S	S	R	R	G-10A	Ins755GT	none	T271C (S91P)	Lisboa3
FF505	1997	IRSE	nd	nd	nd	nd	G-10A	Ins755GT	none	T271C (S91P)	Lisboa3
BK530	2005	IRSEP	S	S	R	R	G-10A	Ins755GT	Ins1211G	T271C (S91P)	Lisboa3
BK872	2005	IRSEP	R	S	R	R	G-10A	Ins755GT	Ins1512G	T271C (S91P)	Lisboa3
BK30	2005	IRSEP	R	S	R	R	G-10A	Ins755GT	none	T271C (S91P)	Lisboa3
BK81	2005	IRSEP	R	S	R	R	G-10A	Ins755GT	none	T271C (S91P)	Lisboa3
BK127	2005	IRSEP	R	R	R	R	G-10A	Ins755GT	none	T271C (S91P)	Lisboa3
BK214	2005	IRSEP	R	R	R	R	G-10A	Ins755GT	none	T271C (S91P)	Lisboa3
BK309	2005	IRSEP	R	S	R	R	G-10A	Ins755GT	none	T271C (S91P)	Lisboa3
BK548	2005	IRS	R	S	R	R	G-10A	Ins755GT	none	T271C (S91P)	Lisboa3
BK801	2005	IRSE	S	S	R	R	G-10A	Ins755GT	none	T271C (S91P)	Lisboa3
BK839	2005	IRSEP	R	S	R	R	G-10A	Ins755GT	none	T271C (S91P)	Lisboa3
BK1144	2005	IRSEP	R	S	R	R	G-10A	Ins755GT	none	T271C (S91P)	Lisboa3
BK128	2005	IRSEP	R	R	R	R	G-10A	G223T (E75X)	G1158T	A281G (D94G)	Lisboa3
IHMT134	2009	IRSP	nd	S	S	S	none	none	none	C249G	Lisboa3
FF181	1997	IRS	nd	nd	nd	nd	none	none	none	none	Lisboa3
FF291	1998	IRS	nd	nd	nd	nd	none	none	none	none	Lisboa3
FF593	1997	IRS	nd	nd	nd	nd	none	none	none	none	Lisboa3
FF164	1997	IRS	nd	nd	nd	nd	none	none	none	none	Lisboa3
IHMT82	2009	IRS	nd	S	R	S	none	none	none	none	Lisboa3
BK519	2005	IRESP	R	S	R	R	none	none	C1402A; G1484T	A281G (D94G)	Lisboa3
BK87	2005	IRS	R	R	R	R	none	none	G1484T	A281G (D94G)	Lisboa3
HVNG1	2008	IRSEP	R	R	R	S	none	none	A1401G	A209G (H70R)	NC
FF291	1997	IRE	nd	nd	nd	nd	none	none	none	G280A(D94N)	NC
HVNG2	2009	IRS	S	S	S	S	none	none	none	none	NC
HPV113	2008	IRSEP	S	S	S	S	none	none	none	none	NC
IHMT149	2009	IRSEP	nd	R	R	R	none	none	A1401G	A281C (D94A)	Q1
IHMT151	2009	IRSEP	nd	R	R	R	none	none	A1401G	A281C (D94A)	Q1
HPV115	2008	IRSEP	R	R	R	R	none	none	A1401G	A281C (D94A)	Q1
IHMT361	2008	IREP	nd	S	R	S	none	none	none	none	Q1
BK104	2005	IREP	R	R	R	R	none	none	A1401G	A281C (D94A)	Q1
BK269	2005	IREP	R	R	R	R	none	none	A1401G	A281C (D94A)	Q1
BK364	2005	IRSEP	R	R	R	R	none	none	A1401G	A281C (D94A)	Q1
BK460	2005	IREP	R	R	R	R	none	none	A1401G	A281C (D94A)	Q1
BK474	2005	IRSEP	R	R	R	R	none	none	A1401G	A281C (D94A)	Q1
BK667	2005	IREP	R	R	R	R	none	none	A1401G	A281C (D94A)	Q1
BK733	2005	IREP	R	R	R	R	none	none	A1401G	A281C (D94A)	Q1
BK396	2005	IRSEP	R	R	R	R	none	none	A1401G; G1158T	A281C (D94A)	Q1
BK372	2005	IR	R	R	R	R	none	none	none	none	Q1

<sup>a</sup>First-Line DST, I – isoniazid; R – rifampicin; E – ethambutol; S – streptomycin; P – pyrazinamide.<sup>b</sup>Second-line Resistance, R – Resistant, S – Susceptible, nd – not determined.<sup>c</sup>Nucleotidic mutations and, in brackets, aminoacidic mutations.



Comparing the AMK and KAN phenotypic resistance profiles we have observed that from the 17 isolates with second-line DST data bearing the *eis* G-10A mutation, nine were resistant to KAN alone; four were resistant to both KAN and AMK and four were susceptible to either (Table 5.1).

This observation opened the question concerning the molecular basis of AMK resistance when no *rrs* mutations are found. According to the published work by Zaunbrecher *et al*, *eis* promoter mutations may cause resistance to KAN but not AMK.<sup>372</sup> We have found however that isolates bearing *eis* G-10A mutations might be KAN and AMK resistant, KAN resistant only or susceptible to both. One hypothesis is that the level of KAN or AMK resistance is lower than the level of resistance conferred by *rrs* mutations. This low-level resistance if close enough of the tested critical concentration might yield discrepant results independently of its genotype but dependent on the methodology employed in drug susceptibility testing. To investigate this hypothesis, we have determined the KAN and AMK MIC for selected isolates bearing different mutations using the REMA methodology (Table 5.2). We have found that isolates bearing the *rrs* A1401G mutations all exhibited a high-level resistance (>80 mg/L) to both AMK/KAN. The results obtained for the two isolates bearing the *eis* G-10A mutation yielded slightly different results, with one isolate exhibiting an MIC of 5 mg/L for KAN (equal to the critical concentration used in the MGIT™ 960 system) and 0.63 mg/L (below the critical concentration used in the MGIT™ 960 system) for AMK. The other isolate exhibited KAN and AMK MICs (10 and 1.25 mg/L, respectively) above the critical concentrations used in KAN/AMK DST (Table 5.2). Both isolates initially tested susceptible to AMK/KAN (Table 5.1), which may corroborate the proposed hypothesis that *eis* G-10A mutations may cause low-level resistance and discrepant DST results, especially in the case of AMK DST. It is important to stress the fact that the REMA methodology is susceptible to phenotypic variations and presents difficulties in obtaining accurate readings at low level concentrations.<sup>483</sup> Nevertheless, in all plate assays the *M. tuberculosis* H37Rv was used as a susceptible control strain and used to establish the baseline MIC for comparison of resistance levels.

In the *gyrA* gene, four different mutations were found, three of which occurring in codon 94. All mutations detected in this gene were missense mutations and were cluster associated. D94A mutation occurred only in Q1 cluster, whereas S91P and D94G were associated only with the Lisboa3 cluster. A non-clustered isolate recovered in 1997 with an unknown second-line resistance profile was found to have mutation D94N, previously associated with FQ resistance, although no second-line susceptibility results were available for this strain. The H70R mutation

found in another non-clustered isolate (HVNG1) could not be associated with FQ resistance since the isolate is susceptible to OFX.

**Table 5.2** – KAN and AMK MIC for selected strains bearing the *rrs* A1401G and *eis* G-10A mutations.

Isolate	Year	Mutations		MIC (mg/L)	
		<i>rrs</i>	<i>eis</i>	KAN	AMK
H37Rv	-	none	none	1.25	0.63
HPV129	2009	none	G-10A	10	1.25
HPV108	2009	none	G-10A	5	0.63
HPV115	2008	A1401G	none	>80	>80
HVNG1	2008	A1401G	none	>80	>80
IHMT149	2009	A1401G	none	>80	>80
IHMT151	2009	A1401G	none	>80	>80

#### Genotypic comparison reveals clonality and resistance acquisition dynamics.

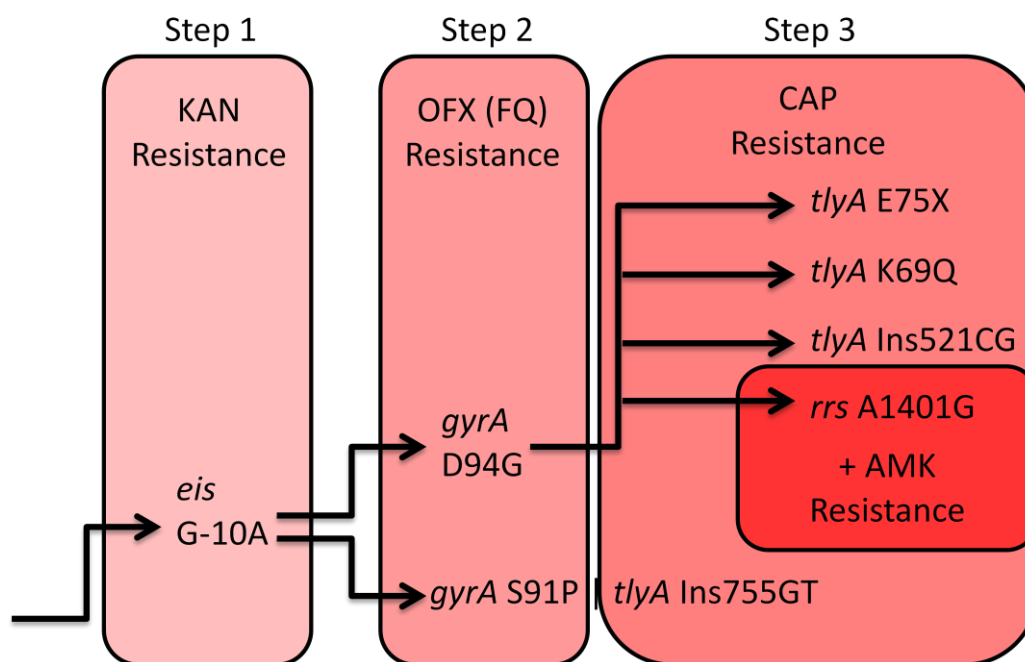
The overall isolate distribution through Q1 and Lisboa3 clusters allowed the following observations: in Q1 cluster (except for one isolate) one mutation only (*rrs* A1401G) ensures resistance to all three second-line injectable drugs, with no mutation detected in *eis* and *tlyA* genes. Moreover, in Q1 cluster two mutational events, in *rrs* and *gyrA*, were enough for a MDR-TB strain to become XDR in two mutational steps. With the current panel of isolates it is impossible to determine which resistance was acquired in the first place since all isolates with the *rrs* A1401G mutation also have the *gyrA* D94A mutation.

In Lisboa3 cluster, however, it is possible to establish the dynamics of resistance acquisition. The mutational analysis itself allows the discrimination of the entire cluster as shown by the intra-cluster mutational diversity. We have observed that whenever a mutation was present in *tlyA* gene, no classical *rrs* mutation (A1401G, C1402A/T or G1484T) was detected and, that isolates belonging to Lisboa3 cluster with a *tlyA* mutation also had an *eis* mutation (G-10A) (Table 5.1).

## 5.5 DISCUSSION

In the present study, we have analyzed the second-line drug resistance associated genes in 44 MDR/XDR-TB clinical isolates, of which 40 belong to two different highly-prevalence clusters. Lisboa3 cluster was first described in the 90s already associated with MDR, and later XDR, while Q1 cluster was described in 2008 as a more homogeneous cluster, from a mutational standpoint and, already associated with MDR and XDR.<sup>24-26</sup>

Looking at the second-line resistance profiles, specifically, resistance to KAN, AMK and CAP, we found that in Q1 cluster (except for one isolate) the acquisition of resistance to these three injectable second-line drugs was caused by a single mutational event (*rrs* A1401G); however, in Lisboa3 cluster our data suggests that resistance to KAN/AMK was first acquired through an *eis* hypermorphic mutation and, at a later stage to CAP due to *tlyA* mutations. Therefore, both genetic clusters illustrate single and multistep processes of resistance development by strains that were subsequently transmitted in the community generating primary XDR-TB cases. Such observations allow the establishment of a putative model for the resistance acquisition pattern in Lisboa3 cluster (Figure 5.1). This model consists in a multistep process more extensive than the one observed in the Q1 cluster. We propose that in Lisboa3 cluster an MDR strain initially acquired resistance to KAN, followed by FQ resistance acquisition, twice and independently, followed by CAP resistance acquisition through multiple events and finally, acquisition of a higher level of resistance to AMK, KAN and CAP through an *rrs* A1401G mutation. The only strain (FF359) to exhibit this later step of resistance acquisition was isolated in 1998 and its dissemination was most probably contained since no Lisboa3 isolate with a *rrs* A1401G was found in recent years. For the Lisboa3 strains bearing the *gyrA* S91P and *tlyA* Ins755GT mutations it is impossible to determine if FQ resistance was acquired first than CAP resistance or vice-versa since all strains with one of these mutations also have the other. Noteworthy is the fact that 2 out of 7 strains isolated between 1997-1998 and involved in the original Lisbon MDR-TB outbreak described by us, already had at the time the mutational profile of recent circulating strains.<sup>26</sup> Second-line DST is not available for these strains but the fact that some isolates exhibit the Lisboa3 characteristic *rrs*, *tlyA* and *gyrA* mutations indicate that they were most likely XDR-TB strains at that time. This highlights the ancient XDR-TB problem that is present in Lisbon Health Region for almost two decades.



**Figure 5.1** – Multistep process of resistance acquisition dynamics in Lisboa3 cluster. The scheme represents the process through which XDR has most likely been acquired multiple independent times in a maximum of three steps. Step 1 consisted in the acquisition of KAN low-level resistance through an *eis* G-10A mutation, followed by the acquisition of FQ resistance by a *gyrA* D94G or S91P mutation, although regarding the strains with the S91P mutation it is impossible to ascertain whether FQ resistance or CAP resistance was acquired in the first place. Strains with the *gyrA* D94G mutation acquired CAP resistance multiple times through different mutations in *tlyA* gene. One strain was found to have acquired CAP and KAN resistance through a *rrs* A1401G mutation, thus strengthening the KAN resistance.

The pattern and the proposed resistance acquisition dynamics described above illustrate the need for a more efficient MDR/XDR-TB management in two different aspects. First, the need to contain specific clusters or families of strains associated with MDR/XDR-TB, such containment should be supported in a better contact tracing for affected individuals and more rapid and reliable diagnostics. Given the time-consumable culture and DST of *M. tuberculosis*, several rapid molecular diagnosis systems have become available. However, MDR-TB molecular diagnosis *per se* is of no use if no information regarding susceptibility to second-line drugs is available. Starting an empirical or standardized treatment for MDR-TB may result in further drug resistance development, and such is highlighted by the dynamics shown in the present study, in which two mutational events are enough for a strain to become XDR. The necessary tools to perform such molecular testing in a setting-adapted approach are sometimes not readily available commercially and still, before implementation of these commercial tests it is necessary to determine the local applicability due to the geographical

variability of resistance-associated mutations. As the body of knowledge concerning new mutations and drug resistance associated genes expand it will be gradually easier to associate both genotype and phenotype.

Another aspect to consider is the issue of mismanagement of drug therapy, poor regimen implementation, poor compliance or default that leads to cumulative resistance acquisition. Although the high prevalence of XDR-TB in the region illustrates the need for new drugs and new drug regimens for MDR/XDR-TB treatment, special care must be taken in the introduction of new drugs in TB treatment.<sup>486</sup> The additional emergence of resistance to new compounds may render these drugs ineffective in a short time period.<sup>487, 488</sup>

Zaunbrecher *et al* proposed that *eis* promoter mutations would be associated with KAN resistance only, and therefore explain the lack of cross resistance between KAN and AMK in some clinical isolates.<sup>372</sup> Although in such cases an increase in the level of AMK resistance was observed, the strains were phenotypically considered susceptible. The question resides in the degree of AMK resistance conferred by *eis* promoter mutations has clinical relevance and impairs chemotherapy and can be detected in drug susceptibility testing? Our data shows that four isolates containing *eis* G-10A mutation and no *rrs* mutations were phenotypically resistant to AMK and KAN. Is it therefore possible that the degree of AMK resistance may be close enough to the critical concentration used in drug susceptibility testing resulting in undetectable low-level resistance?

Furthermore, we have determined the KAN and AMK MIC for selected strains and the data obtained corroborate the hypothesis that the increase in the KAN MIC for *eis* G-10A isolates is accompanied by a slighter AMK MIC increase. A two-fold increase in the AMK MIC, when compared with the AMK MIC for *M. tuberculosis* H37Rv, is not as significant as the increase in KAN MIC but, it may be sufficient to confer border-line/undetectable resistance and hence, explain discrepant or inconsistent results. The same may happen with KAN DST, as one strain exhibited a four-fold increase and another strain an eight-fold increase in KAN MIC. Supporting this, Engstrom *et al* have reported the occurrence of *eis* G-10A mutations among KAN susceptible isolates, which may be due to the low-level resistance conferring MICs close to the critical concentration used in DST.<sup>366</sup> Similarly, Campbell *et al* reported that in 111 KAN-resistant isolates, 32% had *eis* mutations with no isolate accumulating mutations at *rrs* and *eis* simultaneously.<sup>219</sup> Moreover, the authors also reported one isolate resistant to KAN and AMK with an *eis* promoter mutation only. In one other study by Feuerriegel *et al*, 10 AMK and CAP resistant isolates did not show any mutation in the *rrs* and *tlyA* genes and the same was true

for strains resistant to AMK alone.<sup>367</sup> One possible explanation is that AMK resistance in these isolates is mediated by *eis* promoter mutations. The authors however have not tested the isolates for susceptibility to KAN, which would help to clarify this mechanism. In fact, current directives from Portuguese Health Authorities state that second-line DST is not mandatory for KAN, only for AMK and CAP. Nonetheless, as confirmed by this study, it is important to stress that susceptibility to AMK does not imply susceptibility to KAN. In our opinion, KAN DST is of great importance as KAN resistance alone may be associated with undetectable AMK low-level resistance, which may eventually impact on the clinical outcome or resistance development. The non-simultaneous occurrence of mutations at *eis* and *rrs* in more recent isolates, representing a genetic cluster (Lisboa3) circulating for over a decade, suggests that mutations in either of these genes are sufficient to confer phenotypic resistance in the host and impair clinical treatment. Otherwise, we would expect to find at this stage Lisboa3 strains that have acquired AMK and KAN high-level resistance.

Also recently, data from Kim *et al* show that the Eis protein has an important role in the suppression of the host immune system through the acetylation of DUSP16/MKP-7, inhibiting inhibition of JNK-dependent autophagy, phagosome maturation, and reactive oxygen species generation.<sup>489</sup> This Eis important immune modulator property combined with its overexpression might be responsible for an increased virulence phenotype and be a clue to explain the high prevalence and persistence of certain Lisboa family strains in the region.

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## 5.8 TRANSPARENCY DECLARATIONS

None to declare





# **GidB mutation as a phylogenetic marker for Q1 cluster *Mycobacterium tuberculosis* isolates and intermediate-level streptomycin resistance determinant in Lisbon, Portugal**

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## 6.1 ABSTRACT

Development of STP-resistance in *M. tuberculosis* is usually associated with mutations in *rpsL* and *rrs* genes, although up to 50% of clinical STP-resistant isolates may present no mutation in either of these genes. In the present report we investigate the role of *gidB* gene mutations in STP resistance.

We have analyzed 52 STP-resistant and 30 STP-susceptible *M. tuberculosis* clinical isolates by sequencing and endonuclease analysis of the *gidB* and *rpsL* genes. All clinical isolates were genotyped by 12-*loci* MIRU-VNTR.

The *gidB* gene of 18 STP-resistant isolates was sequenced and four missense mutations were found: F12L (1/18), L16R (18/18), A80P (4/18) and S100F (18/18). The remaining isolates were screened by endonuclease analysis for mutations A80P in *gidB* and K43R in *rpsL* gene. Overall, mutation A80P in *gidB* gene was found in 7 STP-resistant isolates and 12 STP-susceptible multidrug resistant isolates. Also noteworthy, is the fact that *gidB* mutations were only present in isolates without *rpsL* and *rrs* mutations, all from genetic cluster Q1. STP semi-quantitative drug susceptibility testing showed that isolates carrying the *gidB* A80P mutation were STP intermediate-level resistant and that standard drug susceptibility testing yielded inconsistent results probably due to borderline resistance.

We conclude that *gidB* mutations may explain the high number of STP-resistant strains with no mutation in *rpsL* or *rrs*. These mutations might occasionally confer undetected STP low-level resistance in regular drug susceptibility testing. Also, GidB A80P mutations may serve as surrogate markers for Q1 cluster isolates that are associated with MDR/XDR-TB.

## 6.2 INTRODUCTION

STP is an aminoglycoside antibiotic, the first that was introduced in the treatment of TB, revolutionizing the treatment of this disease. Although mostly regarded as a tuberculostatic agent, STP is no longer a first option in TB standardized treatments for new cases. It is still incorporated as first-line TB therapeutical regimens in patients that have previously been treated for TB (former WHO Category II “retreatment” regimen) and in some countries incorporated in second line regimens for drug-resistant TB, although it’s not the first choice for injectable aminoglycosides given the high rates of STP resistance in drug-resistant TB.<sup>203, 490</sup>

In spite of this, due to the global emergency of resistant *M. tuberculosis* strains, such as MDR and XDR strains, STP may regain an important role in TB management.

Portugal has an intermediate incidence rate when compared with other countries of the European Union with 2 398 new cases (22.5 new cases per 100 000 habitants), in 2010, but a worrying situation regarding M/XDR-TB.<sup>21, 24-26, 482</sup> The latest TB surveillance data available and published by the ECDC for STP resistance reported, in 2008, a 9.5% prevalence of STP resistance (156 cases out of 1641 cases with DST results), which exceeded other antibacillary drug resistance prevalence’s such as INH (7.4%) and RIF (1.8%).<sup>491</sup>

STP inhibits protein synthesis through irreversible binding to the A-site of the 30S ribosomal subunit and to the ribosomal protein S12. Ribosomes combined with STP are unable to initiate and elongate during the process of gene translation. STP also prevents dissociation of ribosomal 50S and 30S subunits.<sup>312, 323</sup>

Accordingly with its mode of action, STP resistance is usually mediated by mutations in *rpsL* and *rrs* genes, which encode the ribosomal protein S12 and the 16S rRNA, respectively.<sup>312</sup> The most common mutation occurs in codon 43 of the *rpsL* gene and can be detected by PCR-RFLP with *MbolI*.<sup>463</sup> Mutations conferring STP resistance in *rrs* have also been described, usually in the 530 and 912 loop regions, and since *M. tuberculosis* only possesses one copy of the rRNA operon, one mutation in this gene is enough to cause resistance.<sup>312, 492</sup> More recently, a new gene, *gidB*, encoding a rRNA methyltransferase was implicated in STP resistance.<sup>325</sup> The gene product of *gidB* is involved in the methylation of 16S rRNA, more specifically, at position G527. Since binding of STP to 16S rRNA is crucial to its action mechanism, mutations in *gidB* may well be responsible and explain STP resistance when no mutation is found on *rpsL* or *rrs*.<sup>325</sup> Notably, in the STP-resistant strains isolated in our laboratory, *rrs* mutations that confer STP resistance were never detected in the STP-resistant strains lacking *rpsL* mutations.<sup>24</sup>

The *gidB* gene is highly conserved in all eubacteria, including *Mycoplasma genitalium*, and loss of function has been implicated in high-level STP resistance in *Salmonella* but with low-level resistance in *M. tuberculosis*.<sup>435, 493</sup>

In this study, we screened 52 STP-resistant and 30 STP-susceptible isolates circulating in Lisbon Health Region for mutations in *gidB* gene and its association with STP resistance.

### 6.3 METHODS

#### Clinical Isolates

Two sets of isolates were analyzed in the present study, the first comprised by a total of 82 *M. tuberculosis* isolates, 52 STP-resistant isolates and 30 STP-susceptible (14 multidrug-resistant and 16 susceptible to all first-line drugs), recovered from the same number of patients between 2004-2006. For this set of isolates, only DNA was available. All isolates were collected from hospitals and public health laboratories in Lisbon Health Region.

The second set of isolates is comprised by 6 isolates, 3 STP-resistant and 3 STP-susceptible, and was included in the study to provide data on STP semi-quantitative Drug Susceptibility Testing (qDST).

#### STP susceptibility testing

All isolates were tested for STP susceptibility by the fluorimetric BACTEC™ MGIT™ 960 system (Becton Dickinson Diagnostic Systems, Sparks, MD, USA) using a critical STP concentration of 1,0 mg/L, according to the manufacturer's instructions.

STP qDST was carried out for the selected isolates using three STP concentrations (1.0, 4.0 and 20.0 mg/L) on the MGIT 960 system and the Epicenter V5.80A software equipped with the TB eXIST module (Becton Dickinson Diagnostic Systems™, Sparks, MD, USA) as previously described.<sup>151</sup>

#### PCR amplification and DNA sequencing

The ORF of *gidB*, plus 96 nucleotides upstream and 56 nucleotides downstream, was amplified by PCR resulting in a 828 bp fragment using forward oligonucleotide primer gidBF1, and reverse oligonucleotide primer gidbR1 (Table 6.1). Cycling conditions for this amplification consisted of an initial denaturation step at 94°C for 10 min; 40 cycles of a denaturation step at

94°C for 1 min, a primer annealing step at 67.5°C for 35 sec and, an extension step at 72°C for 1 min; a final extension step at 72°C for 10 min was performed.

The entire *rpsL* ORF, plus 46 nucleotides upstream and 86 nucleotides downstream, was also amplified by PCR resulting in a 504 bp fragment using forward oligonucleotide primer RPSL-1, and reverse oligonucleotide primer RPSL-2 (Table 6.1).<sup>450</sup> Cycling conditions for this amplification consisted of 40 cycles of a denaturation step at 94°C for 1 min, a primer annealing step at 57°C for 2 min and, an extension step at 72°C for 2 min.

The region between nucleotides 10-1037 of the *rrs* gene was amplified by PCR using forward oligonucleotide primer RRS-1 and reverse oligonucleotide primer RRS-2 (Table 6.1).<sup>450</sup> Cycling conditions consisted in 40 cycles of a denaturation step at 94°C for 1 min, primer annealing step at 60°C for 2 min, and an extension step at 72°C for 2 min.

Amplicons were purified with Wizard™ SV Gel and PCR Clean-Up System (Promega™) prior to sequencing. Sequencing reactions were performed with BigDye Terminator Cycle Sequencing Kit with AmpliTaq DNA polymerase (Applied Biosystems™) using oligonucleotide primers: RPSL-1 for *rpsL* amplicons, RRS-1 and RRS-2 for *rrs* amplicons and, gidBF1 and gidBR1 for *gidB* amplicons (Table 6.1).

**Table 6.1** - Oligonucleotides used for amplification and sequencing of the *rpsL* and *gidB* genes.

	Sequence	Tm (°C)	Reference
RPSL-1	5'-GGCCGACAAACAGAACGT-3'	63.8	Kirschner <i>et al</i> <sup>450</sup>
RPSL-2	5'-GTTACCAACTGGGTGAC-3'	58.8	Kirschner <i>et al</i> <sup>450</sup>
RRS-1	5'-GAGAGTTTGATCCTGGCT-3'	62.9	Kirschner <i>et al</i> <sup>450</sup>
RRS-2	5'-TGCACACAGGCCACAAGG-3'	72.5	Kirschner <i>et al</i> <sup>450</sup>
gidBF1	5'-AAGCGATGCGTGGCCGAGCG-3'	69.4	This study
gidBR1	5'-CTGGCCCGACCTTACGAGCG-3'	68.4	This study

### PCR-RFLP analysis

Mutation A128G in *rpsL* was detected by *MbolI* (New England Biolabs™) digestion of the *rpsL* amplicon. Two micrograms of PCR product were digested with 5U of *MbolI* accordingly to the manufacturer's instructions for 1 h at 37°C. Absence of restriction indicates the presence of mutation A128G.

Mutation G238C in *gidB* was detected as above with the use of *BsrDI* enzyme. The occurrence of restriction indicates the presence of mutation G238C.

### MIRU-VNTR genotyping

All isolates were genotyped by 12-*loci* MIRU-VNTR as described by Supply *et al.*<sup>57</sup> A dendrogram was constructed at MIRU-VNTRplus web application available at <http://www.miru-vntrplus.org> using the Dsw measure of genetic distance and the UPGMA. A cluster was defined as group of more than one isolate sharing the same MIRU-VNTR profile.

### Bioinformatic analysis of GidB mutations

Alignment of GidB aminoacidic sequences was carried out using BioEdit (v.7.0.5.2, T.A. Hall) for orthologous sequences from *M. tuberculosis* H37Rv (GI: 15611055), F11 (GI: 148825127), CDC1551 (GI: 15843553); *Mycobacterium bovis* AF2122/97 (GI: 31795092); *Mycobacterium africanum* GM041182 (GI: 339633908); *Mycobacterium smegmatis* MC<sup>2</sup>155 (GI: 118471658); *Mycobacterium leprae* TN (GI: 15828466); *Mycobacterium avium* subsp. *avium* ATCC 25291 (GI: 254777657); *Mycobacterium kansasii* ATCC 12478 (GI: 240168403); *Corynebacterium diphtheriae* NCTC 13129 (GI: 38234911); *Arthrobacter globiformis* NBRC 12137 (GI: 359776049); *Actinomyces odontolyticus* F0309 (GI: 293191006); *Nocardia brasiliensis* ATCC 700358 (GI: 378817268); *Proteus mirabilis* HI4320 (GI: 197286881); *Yersinia pestis* KIM10+ (GI: 22128011); *Escherichia coli* MS 21-1 (GI: 300940926) and O157:H7 str. EDL933 (GI: 15804340); *Staphylococcus pettenkoferi* VCU012 (GI: 365225940); *Bacillus subtilis* subsp. *subtilis* str. 168 (GI: 16081152); *Mycoplasma genitalium* G37 (GI: 12045241); *Haemophilus influenzae* Rd KW20 (GI: 16272434); *Streptococcus pneumoniae* D39 (GI: 116516833); *Enterococcus faecium* PC4.1 (GI: 293379357); *Neisseria meningitidis* alpha522 (GI: 389604808); *Brucella melitensis* ATCC 23457 (GI: 225853491) and, *Salmonella enterica* subsp. *enterica* serovar Typhi str. M223 (GI: 213850448).

Impact of specific mutations on protein function was assessed based on the degree of conservation of amino acid residues using the SIFT program server available at

<http://sift.jcvi.org/>. Comparison was made using the *GidB* sequence from *M. tuberculosis* H37Rv against the UniRef90 database.<sup>494</sup>

## 6.4 RESULTS

Screening a set of 18 STP-resistant isolates for *gidB* mutations we have found four different missense mutations: F12L, L16R, A80P and S100F. Mutations L16R and S100F were present in all isolates tested. When analyzing the *gidB* homologous genes from both *M. tuberculosis* CDC1551 and F11 we found mutation S100F to be present on both strains and that *M. tuberculosis* F11 also had the L16R mutation. These two mutations were therefore considered natural polymorphisms and were not considered as mutations associated with STP resistance. *GidB* A80P and F12L mutations were detected in 4 and 1 isolates, respectively.

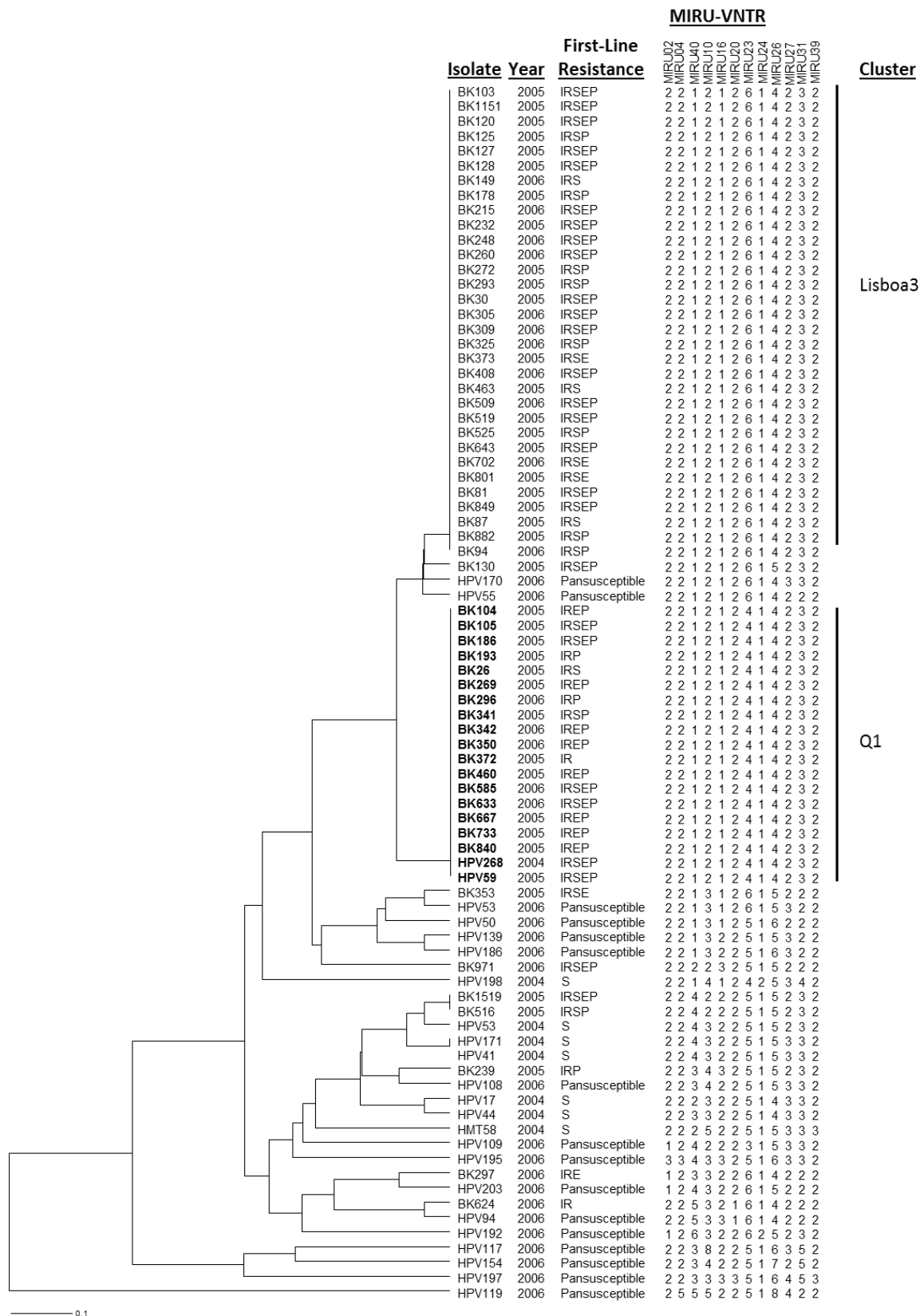
The A80P mutation was therefore considered the most prevalent mutation occurring in the *gidB* gene and a method to rapidly screen for G238C (A80P) mutation in *gidB* through PCR-RFLP was devised through amplicon digestion with *BsrDI*. Thirty-four additional STP-resistant isolates and 30 STP-susceptible isolates were characterized by this manner. The results obtained for each isolate were compared with the characterization of the *rpsL* gene.

Overall, the *GidB* A80P mutation was detected in 19 out of 82 isolates, 7 STP-resistant and 11 STP susceptible. Only MDR isolates were found to bear the A80P mutation, whereas the 16 pan-susceptible isolates included in the study didn't harboured this mutation (Figure 6.1). Also noteworthy, all strains containing the *GidB* A80P mutation lacked *rpsL* and *rrs* mutations.

To examine the distribution of this prevalent mutation and in an attempt to associate this specific mutation with any genetic cluster, family or clade, all isolates were genotyped by 12-*loci* MIRU-VNTR. We found that all isolates carrying the *GidB* A80P mutation belonged to genetic cluster Q1 (Figure 6.1). The occurrence of *GidB* A80P mutation in all Q1 isolates and the fact that this mutation occurs among both STP-resistant and susceptible isolates may suggest that this mutation has no contribution to STP-resistance but may rather be, from a resistance stand-point, a neutral phylogenetic polymorphism characteristic of Q1 isolates. Alternatively, this mutation might constitute an adaptation mechanism acquired by an ancestral Q1 isolate that yielded clinically significant but borderline STP resistance on standard DST.



To further elucidate the contribution of *GidB* A80P mutation to STP resistance, we decided to study the STP qDST profiles in a second set of 6 *M. tuberculosis* clinical isolates composed by 4 Q1 isolates carrying the *GidB* A80P mutation, one Beijing strain carrying the *rpsL* K43R mutation and a pan-susceptible isolate with no mutation in *gidB*, *rpsL* or *rrs*. The results obtained show that the four Q1 isolates were all resistant to 1 mg/L, of which three were resistant to 4 mg/L, while the remaining isolate displayed an intermediate resistance to 4 mg/L (Table 6.2). All Q1 isolates were susceptible to 20 mg/L. The Beijing strain with the K43R mutation on the *rpsL* gene was resistant to the three tested STP concentrations whereas the pan-susceptible clinical strain and the control strain H37Rv were found to be susceptible to the three tested concentrations (Table 6.2). The results obtained show that the Q1 isolates carrying the *GidB* A80P mutation display an increased STP resistance-level towards an intermediate-level resistance (Table 6.2). Three Q1 isolates with the *GidB* A80P mutation (two STP susceptible and one resistant) were also blindly retested in duplicate, using standard DST methodology on BACTEC 960 MGIT with a STP breakpoint of 1 mg/L. Surprisingly, the two isolates (IHMT308 and 361) initially considered as STP-susceptible were considered resistant upon retesting (Table 6.2).



**Figure 6.1**– MIRU-VNTR dendrogram of the 82 *M. tuberculosis* clinical isolates screened for *gidB* and *rpsL*. Isolates carrying the GidB A80P mutation are shown in bold. Resistance profile: I, isoniazid; R, rifampicin; S, streptomycin; E, ethambutol; and P, pyrazinamide.

**Table 6.2**– STP qDST for selected strains carrying the *GidB* A80P mutation.

Isolate	Year	First-line Resistance <sup>a</sup>	MIRU-VNTR Cluster <sup>b</sup>	Mutations <sup>c</sup>			STP qDST <sup>d</sup>			STP DST Retest <sup>e</sup>	
				<i>gidB</i>	<i>rpsL</i>	<i>rrs</i>	1 mg/L	4 mg/L	20 mg/L	#1	#2
H37Rv	-	Pan-susceptible	N.C.	None	None	None	S	S	S	nd	nd
HPV65	2008	Pan-susceptible	N.C.	None	None	None	S	S	S	nd	nd
IHMT295	2008	IRSEP	N.C. (Beijing)	None	K43R	None	R	R	R	nd	nd
HPV115	2008	IRSEP	Q1	G238C (A80P)	none	None	R	R	S	nd	nd
IHMT308	2008	IRP	Q1	G238C (A80P)	None	None	R	R	S	R	R
IHMT361	2008	IREP	Q1	G238C (A80P)	None	None	R	I	S	R	R
IHMT149	2009	IRSEP	Q1	G238C (A80P)	None	None	R	R	S	R	R

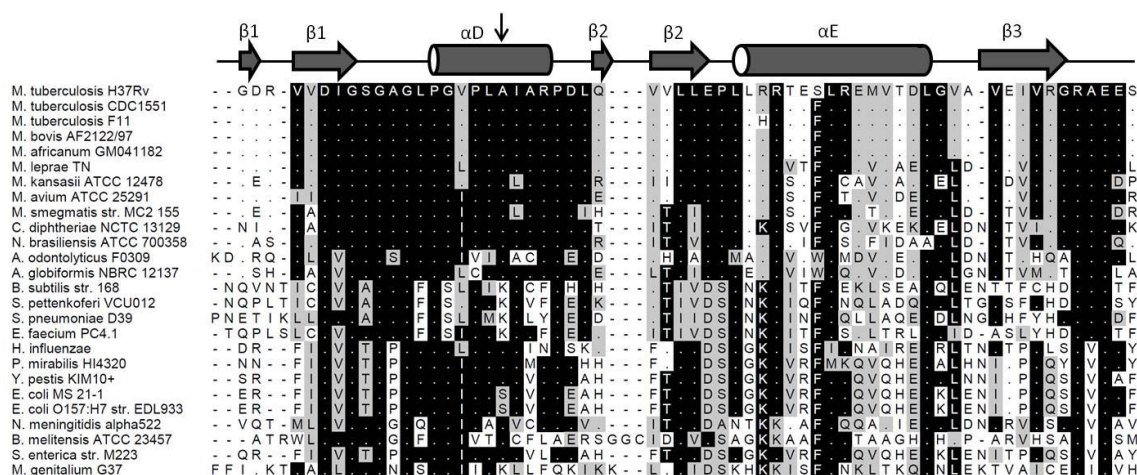
<sup>a</sup> First-Line resistance: I – Isoniazid; R – Rifampicin; S – Streptomycin; E – Ethambutol; P – Pyrazinamide.

<sup>b</sup> N.C. – Non-clustered.

<sup>c</sup> For these isolates the respective genes have been sequenced as described in Material and Methods section. S100F and L16R mutations in *gidB* are omitted.

<sup>d</sup> STP qDST: S – susceptible; I – intermediate; R – Resistant.

<sup>e</sup> STP DST Retest: R – Resistant; S – Susceptible; nd – not determined.



**Figure 6.2** - Alignment of *GidB* homologous sequences from different bacterial species showing conservation of Alanine 80 (vertical arrow) and positioning in the alpha-helix D deduced from the crystal structure of *E. coli* *GidB* (RCSB Protein Data Bank ref. 1JSX).<sup>495</sup> Dots (.) represent identical amino acid residues and alignment gaps are represented by dashes (-). White, gray and black shading represent increasing conservation at each position, respectively.

Analysis of the amino acidic sequence of GidB and comparison with other GidB homologous sequences from other *M. tuberculosis* strains, other mycobacteria species and different bacterial species showed that the GidB alanine 80 is highly conserved among mycobacteria and that some degree of conservation exists among both Gram-positive and –negative bacteria, in particular among Gram-negative (Figure 6.2). Furthermore, the GidB A80P mutation was predicted to affect protein function by SIFT score analysis, yielding a SIFT score of 0.01, resulting from the analysis of 29 sequences.

## 6.5 DISCUSSION

In the present study, we have analyzed the *gidB* gene, encoding a rRNA methyltransferase, in STP-resistant and susceptible isolates. Methyltransferases have demonstrated a growing importance in the action mechanism and resistance pathways to several antimicrobial agents in *M. tuberculosis*. Intrinsic resistance to macrolides is thought to be associated with 23S rRNA methyltransferases encoded by the *erm* genes whereas loss of TlyA methyltransferase confers resistance to cyclic peptides, *e.g.*, CAP. However, most rRNA methyltransferases are still unknown in *M. tuberculosis*.<sup>496</sup>

The screening of *gidB* mutations revealed that besides two naturally occurring polymorphisms (L16R and S100F), two different missense mutations were found, F12L and A80P, the latter being the most prevalent. Both mutations had never been described before and, were only detected in isolates without *rpsL* and *rrs* mutations. As a result, *gidB* mutations may account for STP resistance in isolates with both wild-type *rpsL* and *rrs*. Nevertheless, the A80P mutation was detected in MDR isolates susceptible to STP but not in pan-susceptible isolates.

The A80P mutation has not, to our extent of knowledge, been previously described and bioinformatic analysis revealed that this mutation occurs in the alpha-helix D close to the S-adenosyl-L-methionine binding consensus sequence, according to homology analysis with *E. coli* GidB and respective crystal structure.<sup>495</sup> Moreover, this substitution does not appear to be chemically conservative and may eventually hamper the functionality of GidB in methylating the 16S rRNA leading to an increase in STP resistance level. This latter hypothesis was further supported by SIFT score analysis, which predicted that the A80P mutation has a probable impact on GidB function and consequently on the development of STP resistance.<sup>325</sup>

Other studies have reported the occurrence of *gidB* mutations in susceptible isolates and this phenomenon appears to be related with STP low-level resistance.<sup>214, 497</sup> STP qDST revealed that the majority of tested strains carrying the A80P mutation exhibit an intermediate STP resistance level (*i.e.*, either resistant or intermediate resistance to 4 mg/L but susceptible to 20 mg/L) which may correlate with the STP low-level resistance reported by others relatively to other *gidB* mutations in *M. tuberculosis* clinical isolates.<sup>327, 435</sup> It is possible that strains carrying this mutation may occasionally yield inconsistent DST results as demonstrated by the STP retesting of selected isolates. In the present study the use of the novel qDST methodology takes into account the growth rate in both drug-containing and –free media to establish intermediate resistance levels relative to a specific concentration, rather than just resistant or susceptible as in the traditional MGIT methodology.<sup>151</sup>

Our results indicate that a mutation in *gidB* may be enough to confer clinically relevant STP resistance, although we do not discard a possible contribution of efflux mechanisms to the overall STP resistance level as proposed by Spies *et al* when studying the synergistic effect between *gidB* mutations and efflux pump inhibitors.<sup>326</sup>

The association with the *GidB* A80P mutation with the genetic cluster Q1, a highly prevalent cluster of M/XDR-TB isolates in Lisbon, Portugal, is important since it allows the use of this mutation as a surrogate marker for Q1 isolates and can be useful for its detection. The detection of these isolates may be useful in managing M/XDR-TB in the region since early detection of Q1 isolates would prove valuable for the design of adequate treatment regimens and patient isolation.<sup>24, 25, 482</sup>

Other mutations may be useful for assignment to *M. tuberculosis* lineages. For example, the L16R substitution is associated with isolates from the LAM lineage, which includes the *M. tuberculosis* F11 strain.<sup>214, 327</sup> In fact, approximately 50% of the strains circulating in the Lisbon Health region belong to the LAM lineage of which 1.67% represent Q1 isolates belonging to the LAM4 sub-lineage (unpublished data).<sup>498</sup> The phylogenetic association of the *GidB* S100F polymorphism is, however, unclear as it was present in all the 18 isolates.

Given the high resistance rates in this and other settings, the correct assessment of STP resistance level and eventual association with a molecular marker can be useful in the decision of whether STP should be used in some therapeutic regimens.

Nevertheless, it is also important to stress that the addition of STP alone to a treatment regimen such as the former WHO recommended Category II regimen is counterproductive as it

may lead to the acquisition of STP resistance and, amplification of resistance to other antibacillary drugs in use.<sup>203, 499</sup> A recently published study performed in Peru by Ponce *et al* showed that the cure rate was lower for WHO Category II regimen compared to treatment with Category I regimen. According to the authors, this should support the phasing-out of WHO Category II regimen.<sup>500</sup> Additional data from other countries seem to also support this notion.<sup>501, 502</sup>

In conclusion, our results suggest that the cluster-specific *gidB* polymorphism A80P is responsible for an intermediate level of STP resistance, which has implications for routine STP DST testing as currently performed using a single critical concentration; STP prescription; and, M/XDR-TB detection. Our data, further contributes to global awareness of the role of *gidB* mutations in the process of STP resistance development.

## **6.6 FUNDING**

This work was partially supported by Project Ref. SDH49: “Early Molecular Detection of M/XDRTB in the Great Lisbon Healthcare Region” from Fundação Calouste Gulbenkian (FCG, Portugal). J. Perdigão, D. Machado and C. Silva were supported by FCT grants SFRH/BD/45388/2008, SFRH/BD/65060/2009 and SFRH/BD/73579/2010, respectively.

## **6.7 COMPETING INTERESTS**

The authors have nothing to declare.

## **6.8 ETHICAL APPROVAL**

Not required.

## CHAPTER 7

# **Unraveling *Mycobacterium tuberculosis* genomic diversity and evolution in Lisbon, Portugal, a highly drug resistant setting**

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## 7.1 ABSTRACT

MDR and XDR-TB present a challenge to TB control and elimination goals. In Lisbon, Portugal, particular and successful XDR-TB strains have been found in circulation for almost two decades.

In the present study we have genotyped and sequenced the genomes of 56 *M. tuberculosis* isolates recovered mostly from Lisbon. While genotyping data revealed three major clusters associated with MDR-TB, two of which associated with XDR-TB, the genomic data contributed to elucidate the phylogenetic positioning of circulating MDR-TB strains, showing a high predominance of a single SNP cluster group 5. Furthermore, a genome-wide phylogeny analysis from these strains, together with 19 publicly available genomes of *M. tuberculosis* clinical isolates, revealed two major clades responsible for M/XDR-TB in the region: Lisboa3 and Q1

The data presented by this study contributes to the expanding knowledge of *M. tuberculosis* genomic diversity yielding insights on microevolution and identification of novel compensatory mutations associated with RIF resistance in *rpoB* and *rpoC*. The screening for other structural variations revealed putative clade-defining variants. One deletion in PPE41, found among Lisboa3 isolates, is proposed to contribute to immune evasion and as a selective advantage. IS mapping has also demonstrated the role of *IS6110* as a major driver in mycobacterial evolution by affecting gene integrity and regulation.

The analysis of non-synonymous/synonymous ratios revealed heterogeneities across the chromosome, genotype and Clusters of Orthologous Groups, highlighting possible and different evolution strategies. Globally, our data supports the notion of a growing genomic diversity facing both setting and host adaptation.

## 7.2 INTRODUCTION

TB is responsible for approximately 1.4 million deaths each year and is considered a Global Health Emergency by the WHO. Portugal is the Western European country that over the last few decades has had one of the highest TB notification rates in Europe (24.7 cases per 100 000).<sup>21</sup> Although this rate is considered intermediate, the difficulty is the growing threat of drug resistance. In particular, the two most difficult-to-treat forms: MDR-TB (resistance to the two most powerful first-line drugs – INH, and RIF and XDR-TB (MDR plus resistance to a FQ and a second-line injectable drug).<sup>20, 503</sup>

The TB situation in the capital city, Lisbon (incidence 31.5 cases / 100 000 in 2010) has been extensively studied. Laboratory data on resistance prevalence point to high XDR-TB rates in the region, which in recent years have ranged between 44.3-66.1% of the MDR-TB clinical isolates.<sup>482</sup> Genotyping studies using RFLP-IS6110 and more recently, through the characterization of MIRU-VNTR, have led to the identification of a family of close genetic clusters: the Lisboa family, highly associated with MDR- and XDR-TB.<sup>24, 26, 405</sup> The prevalence of this family in the region may account to up to 74.0% and 80.0% of MDR- and XDR-TB cases, respectively.<sup>24, 25, 482</sup> Another genetically close and endemic cluster, named Q1 also plays an important role in MDR- and XDR-TB in the region and its impact on public health and drug-resistant TB in the region has been addressed in previous publications.<sup>25, 482</sup>

The etiologic agents of TB are the bacterial (sub)species belonging to MTC, such as *M. tuberculosis* or *M. bovis*.<sup>2, 84</sup> *M. tuberculosis* has been regarded for many years as a genetically monomorphic pathogen. Nevertheless, the high-throughput genomic sequencing of diverse clinical strains has revealed a higher degree of variation than initially anticipated.<sup>52, 426, 504, 505</sup> Next-Generation Sequencing (NGS) technology is allowing new insights on the mode of transmission and evolution of the MTC.<sup>428, 430</sup> Furthermore, the ability to compare, at the genomic level, identical strains in different stages of resistance acquisition can also provide new data on the genomic adaptation and compensation to the fixation of resistance-associated mutations in the host's bacilli population.<sup>427, 430</sup>

The genomic determinants of this family of strains are yet to be characterized. In the present study, we have genotyped and sequenced the genomes of 56 *M. tuberculosis* clinical isolates (sourced from the Lisbon Health Region) with the aim of gaining insights into the genomic diversity and microevolution of prevalent MDR- and XDR-TB circulating strains in the Lisbon region.

### 7.3 RESULTS

Of 56 *M. tuberculosis* isolates studied, 36 (64.3%) were resistant to INH and RIF and were therefore classified as MDR-TB isolates, of which we were able to determine the resistance to second-line drugs for 24 isolates. In total, 10 MDR-TB isolates were also classified as XDR-TB (Table 7.1).

#### Genotypic analysis

The 24-*loci* MIRU-VNTR genotyping technique grouped the MDR-TB isolates into three major clusters: Lisboa3-A, Lisboa3-B and Q1 (Figure 7.1). Use of the 12-*loci* set groups Lisboa3-A and -B in a single cluster (Lisboa3, data not shown). Only the Lisboa3-B and Q1 clusters were found to be associated with XDR-TB isolates. Eight of the ten XDR-TB isolates belonged to either Lisboa3-B or Q1 cluster, and one of remaining two strains was found to be Q1-related, raising the possibility of ancestral Q1 XDR followed by posterior divergence from this cluster. No XDR-TB isolate was found to belong to Lisboa3-A cluster, indicative that a strain-specific subset is actually responsible for the Lisboa3 associated XDR-TB in the region.

#### Genomic analysis

The genetic diversity as assessed by MIRU-VNTR can however underestimate the true genomic diversity present in a given setting. All 56 clinical isolates underwent whole genome sequencing (WGS, Illumina HiSeq 2000 technology, paired end 100-150bp). Raw sequencing data were mapped to the H37RV reference, yielding high coverage data for all isolates (mean read depth per position, mean 249.9, range 44 - 1411 fold; mean 99.1% genome covered, range 98.6 - 99.9%) (Table 7.1). The total number of identified SNPs (point mutations differing from H37Rv) ranged between 488-1465 (mean: 928.0, 26.7% in non-coding regions) (Table 7.1). Of the SNPs on coding regions, 58.5% were considered non-synonymous substitutions yielding a mean non-synonymous/synonymous ratio ( $N_s/S$ ) of 1.41 (Table 1). AG, CT, GA and TC transitions were found to be the most frequent substitution types (Supplementary Figure S7.1), which is reflected by a mean transversion/transition ratio ( $T_v/T_s$ ) of 0.62. Overall, across the 56 clinical isolates and 19 publicly available reference strains (F11, CDC1551, KZN1435,

KZN4207, KZN605, KZN\_R506, KZN\_V2475, UT205 RGTB327, RGTB423, CCDC5180, CCDC5079, CTRI-2, BTB05\_552, BTB05\_559, S96\_129, HN878, R1207, and X122), 9419 genome-wide SNPs were identified by mapping to the reference genome of *M. tuberculosis* H37Rv. The number of small insertions and deletions (indels) detected upon read mapping ranged between 15-175 indels per isolate with a size between 1-59 bp (Table 7.1).

### **Global phylogenetic analysis using WGS**

Using WGS data, the 56 clinical isolates and 19 publicly available strains were assigned into established six SCGs and three PGGs.<sup>52, 64</sup> Overall, at least one isolate belonging to each SCG and subgroups was included in the subsequent analysis. Forty-four (78.6%) of the 56 clinical isolates belonged to SCG 5, reflecting the high prevalence of these strains in Lisbon Health Region (Table 7.1).

A phylogenetic tree was inferred from a set of 9419 genome-wide SNPs (Figure 7.2). It reveals that the two main genetic clusters associated with XDR-TB in the region, Q1 and Lisboa3, constitute two genetically close but distinct clades within the SCG 5. The MIRU-VNTR Lisboa3-A cluster was found to form a monophyletic group within the Lisboa3 clade. The MIRU-VNTR Lisboa3-B clade designation was therefore considered as paraphyletic in the light of a genome-wide SNP phylogeny. The sequenced strain closest to the Lisboa3-Q1 clade is *M. tuberculosis* UT205, a virulent Colombian isolate that according with the present phylogeny shares a more recent common ancestor with Q1 strains than with Lisboa3 strains. The KZN strains, responsible for the XDR-TB outbreaks in South Africa, and belonging to the same SCG as Lisboa3 and Q1 strains, are phylogenetically distinctive, forming a monophyletic clade.

**Table 7.1** - Isolate characteristics: DST and data derived from WGS including mapping indicators.

Isolate	DST <sup>a</sup>		SNPs							INDELs <sup>b</sup>		Mapping Indicators <sup>c</sup>				
	First-Line	Second-Line (SL)	SCG	PGG	Non-synonymous mutations (N <sub>s</sub> )	Synonymous mutations (S)	Total in Coding Regions (T <sub>c</sub> )	Total in Non-Coding Regions	Total	N <sub>s</sub> /S Ratio	T <sub>c</sub> /Total	Total	Size Range	Mean Read Depth	Coverage (%)	
ARS10348	IRS	ETH	5	2	410	296	706	243	949	1.3851	0.5865	0.7439	96	1-24	135.18	98.86
ARS11131	IRSP	CAP AMK OFX MOX ETH	5	2	381	286	667	237	904	1.3322	0.6308	0.7378	95	1-24	101.56	98.89
ARS11285	IRS	AMK OFX MOX	4	2	429	302	731	283	1014	1.4205	0.6031	0.7209	114	1-37	159.38	99.83
ARS11463	I	nd	5	2	382	255	637	241	878	1.4980	0.5575	0.7255	76	1-24	52.42	99.47
ARS11661	IS	nd	5	2	378	284	662	234	896	1.3310	0.6443	0.7388	96	1-24	110.16	98.84
ARS12740	IRSP	ETH	5	2	392	287	679	239	918	1.3659	0.6456	0.7397	95	1-24	97.66	98.81
ARS1717	IRP	OFX ETH	6a	3	263	158	421	160	581	1.6646	0.5651	0.7246	67	1-27	68.89	99.76
ARS1760	I	nd	5	2	379	262	641	242	883	1.4466	0.5725	0.7259	83	1-53	69.23	99.40
ARS1900	IRSEP	CAP KAN OFX ETH	5	2	414	301	715	250	965	1.3754	0.6009	0.7409	101	1-24	148.85	98.75
ARS1930	IRSP	na	5	2	389	268	657	228	885	1.4515	0.6158	0.7424	90	1-52	90.43	98.81
ARS2061	IRP	CAP AMK KAN OFX ETH CS PAS	5	2	379	283	662	239	901	1.3392	0.6346	0.7347	89	1-24	80.51	98.83
ARS2202	IRSP	OFX ETH CS	5	2	404	284	688	243	931	1.4225	0.5889	0.7390	91	1-24	79.46	98.99
ARS2573	I	nd	5	2	400	277	677	231	908	1.4440	0.6250	0.7456	97	1-49	82.71	99.03
ARS3649	IRSEP	KAN OFX ETH	5	2	399	282	681	242	923	1.4149	0.5814	0.7378	91	1-24	72.67	98.92
ARS3806	I	nd	5	2	386	283	669	233	902	1.3640	0.6277	0.7417	85	1-24	195.56	98.84
ARS4857	IRP	na	5	2	381	279	660	234	894	1.3656	0.6418	0.7383	92	1-24	91.12	98.86
ARS5858	IREP	OFX	5	2	395	284	679	243	922	1.3908	0.6401	0.7364	102	1-24	142.10	98.98
ARS6483	IRSEP	OFX ETH	5	2	406	303	709	246	955	1.3399	0.5839	0.7424	98	1-24	92.22	98.79
ARS6539	IS	nd	5	2	407	303	710	249	959	1.3432	0.5933	0.7404	63	1-24	273.98	98.87
ARS6559	I	nd	5	2	394	292	686	233	919	1.3493	0.5953	0.7465	86	1-24	63.55	98.76
ARS7496	IS	nd	2	1	589	410	999	405	1404	1.4366	0.6404	0.7115	175	1-39	109.62	99.30
ARS7571	I	nd	5	2	388	265	653	244	897	1.4642	0.5749	0.7280	93	1-24	123.02	99.48
ARS7860	IS	nd	5	2	378	279	657	232	889	1.3548	0.6318	0.7390	86	1-24	72.59	98.60
ARS7884	IRSEP	OFX ETH	5	2	409	300	709	244	953	1.3633	0.5874	0.7440	100	1-24	177.31	98.81
ARS8437	IRSP	CAP ETH	5	2	405	296	701	247	948	1.3682	0.6005	0.7395	92	1-24	179.07	98.78
ARS8600	I	nd	5	2	407	291	698	238	936	1.3986	0.6262	0.7457	74	1-49	254.44	98.97
ARS9427	I	nd	3a	1	632	411	1043	422	1465	1.5377	0.5773	0.7119	152	1-36	44.07	99.82
FF181_97	IRS	na	5	2	417	300	717	249	966	1.3900	0.6185	0.7422	24	1-9	772.29	98.99
FF291_98	IRS	na	5	2	416	294	710	250	960	1.4150	0.6063	0.7396	31	1-18	658.49	98.97
FF359_98	IRS	na	5	2	419	293	712	254	966	1.4300	0.5946	0.7371	31	1-18	836.93	99.01
FF674_96	Susceptible	na	4	2	428	323	751	276	1027	1.3251	0.6047	0.7313	34	1-33	961.69	99.76
HCC1095_10	IRE	nd	3b	2	429	314	743	292	1035	1.3662	0.5577	0.7179	119	1-24	185.45	99.61
HCC1276_11	IRSEP	CAP AMK MOX OFX ETH	5	2	392	281	673	243	916	1.3950	0.6350	0.7347	96	1-24	159.36	98.81

Table 7.1 (cont)

DST <sup>a</sup>				SNPs				INDELs <sup>b</sup>		Mapping Indicators <sup>c</sup>					
Isolate	First-Line	Second-Line (SL)	SCG	PGG	Non-synonymous mutations (N <sub>s</sub> )	Synonymous mutations (S)	Total in Coding Regions (T <sub>c</sub> )	Total in Non-Coding Regions	N <sub>s</sub> /S Ratio	T <sub>c</sub> /T <sub>s</sub> Ratio	T <sub>c</sub> /Total	Total	Size Range	Mean Read Depth	Coverage (%)
HCC1470_11	IRSEP	CAP AMK KAN OFX MOX ETH CS PAS	5	2	412	295	707	251	958	1.3966	0.5830	99	1-24	96.90	98.76
HCC759_09	IR	ETH	2	1	623	414	1037	418	1455	1.5048	0.6102	160	1-28	70.73	99.39
HCC916_10	IRSEP	CAP AMK KAN OFX ETH	5	2	373	281	654	238	892	1.3274	0.6044	103	1-24	145.85	98.82
HPV105_09	S	nd	5	2	400	281	681	244	925	1.4235	0.6214	37	1-22	591.50	98.93
HPV113_08	IRSEP	ETH	6a	3	222	126	348	140	488	1.7619	0.5945	62	1-24	167.28	99.94
HPV115_08	IRSEP	CAP AMK KAN OFX ETH	5	2	312	225	537	196	733	1.3867	0.7318	76	1-21	179.43	98.82
HPV157_06	IS	nd	5	2	397	287	684	248	932	1.3833	0.5760	90	1-24	96.25	98.87
HPV50_09	Susceptible	nd	5	2	397	291	688	238	926	1.3643	0.6554	69	1-24	281.12	98.94
HPV51_09	Susceptible	nd	3c	2	420	309	729	276	1005	1.3592	0.5677	29	1-18	632.88	99.60
HPV65_08	Susceptible	nd	6a	3	247	160	407	162	569	1.5438	0.5837	15	1-18	1149.11	99.57
HPV70_09	Susceptible	nd	5	2	380	277	657	224	881	1.3718	0.6343	26	1-45	1410.21	99.65
HVNG1	IRSEP	CAP AMK KAN	5	2	308	225	533	201	734	1.3689	0.7082	76	1-24	154.30	98.78
IHMT134_09	IRSP	RFB ETH	5	2	332	228	560	203	763	1.4561	0.6935	83	1-24	171.49	98.77
IHMT149_09	IRSEP	RFB CAP AMK OFX MOX ETH	5	2	313	226	539	193	732	1.3850	0.6849	75	1-21	168.54	98.81
IHMT194_11	IRSEP	RFB CAP AMK ETH	5	2	382	274	656	236	892	1.3942	0.6318	83	1-24	47.61	98.96
IHMT288_95	IRSP	RFB ETH	5	2	415	304	719	247	966	1.3651	0.6230	98	1-24	197.48	98.84
IHMT295_08	IRSEP	RFB ETH	2	1	528	337	865	358	1223	1.5668	0.7284	127	1-59	181.48	99.18
IHMT308_08	IRP	RFB ETH	5	2	334	228	562	201	763	1.4649	0.7524	76	1-21	205.47	98.79
IHMT359_03	R	nd	5	2	406	289	695	247	942	1.4048	0.6276	31	1-22	727.91	98.93
IHMT361_08	IREP	CAP ETH	5	2	305	232	537	193	730	1.3147	0.6278	70	1-24	191.20	98.80
IHMT69_11	IRSEP	RFB CAP AMK ETH	2	1	609	420	1029	415	1444	1.4500	0.6307	169	1-39	202.92	99.36
IHMT80_11	IRSEP	RFB CAP AMK ETH	5	2	380	277	657	236	893	1.3718	0.6318	87	1-24	63.98	98.95
IHMT82_09	IRS	RFB CAP ETH	5	2	313	221	534	190	724	1.4163	0.6808	81	1-24	121.47	98.69

<sup>a</sup>First-Line: I - isoniazid, R - rifampicin, S - streptomycin, E - ethambutol, P - pyrazinamide, Second-Line: ETH - ethionamide, KAN - kanamycin, AMK - amikacin,

OFX - ofloxacin, MOX - moxifloxacin, RFB - rifabutin, PAS - para-amino salicylic acid, CS - cycloserine.

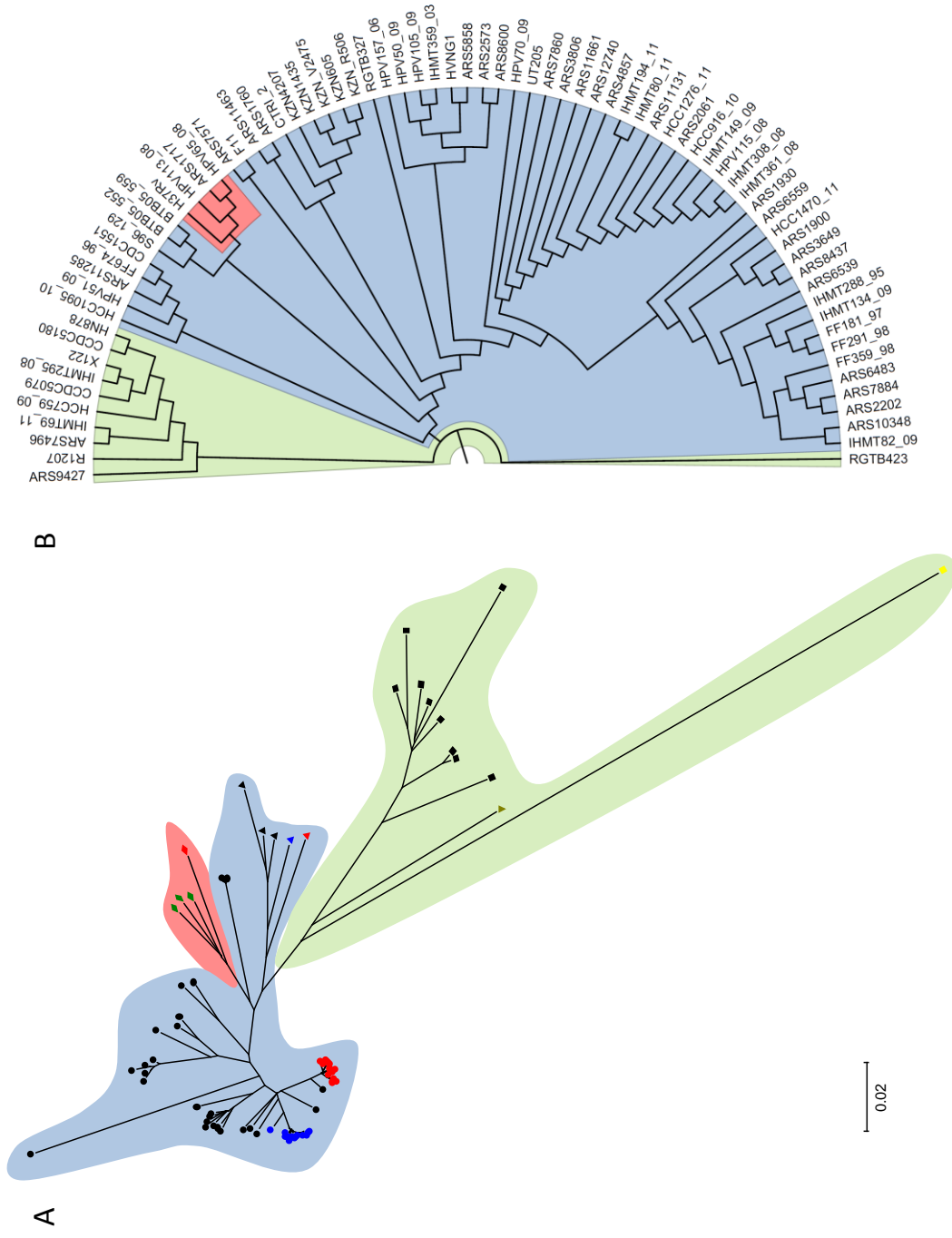
<sup>b</sup>Small INDELs called by SAMtools from mapping to M. tuberculosis H37Rv.

<sup>c</sup>Relative to M. tuberculosis H37Rv

na - not available

nd - not done





**Figure 7.2** – Phylogenetic tree (A) and cladogram (B) of the initial 56 clinical isolates plus 20 *M. tuberculosis* public genomes. PGGs are highlighted in green (PGG1), blue (PGG2) and red (PGG3). A - Isolate-depicting symbols are representative of the different SCGs found in the tree: SCG 1 (yellow square), SCG 2 (black squares), SCG 3a (green triangle), SCG 3b (red triangle), SCG 3c (blue triangle), SCG 4 (black triangle), SCG 5 (circles), SCG 6a (green diamonds), SCG 6b (red diamond). Lisboa3 and Q1 strains are represented by red and blue circles (within SCG 5), respectively.





### Micro-evolution towards multidrug and extensively drug resistance

Given the relative high number of sequenced strains present in both Lisboa3 and Q1 clades it was possible to trace the microevolutionary path reflecting the genomic changes accompanying the resistance acquisition process. We considered the subtrees containing the Lisboa3 and Q1 clades plus one or two strains for the Lisboa3 and Q1 subtrees, respectively, included as outgroups for the ensuing analysis (Figure 7.3). In particular, we inferred the changes in candidate resistant gene mutations at the nodes of the trees.

The Lisboa3 subtree, including the outgroup strain ARS6559, was found to be characterized by a 5 bp deletion on the *iniA* gene. There is a common acquisition of high-level INH resistance through a *inhA* double mutation (in node B). The data also reflect the acquisition of RIF resistance in three separate occasions, twice in the Lisboa3-B strains by a *rpoB* S450L (equivalent to *E. coli* S531L) and in Lisboa3-A lineage by a *rpoB* D435V (equivalent in *E. coli* to D516V). Acquisition of XDR can be seen in the two branches: the first by acquisition of an *eis* G-10A, *gyrA* S91P and *tlyA* Ins755GT mutations (node B1); and, by an *eis* G-10A and *gyrA* D94G mutations (node E1). The latter XDR lineage with a *eis* G-10A mutation will most likely present resistance to KAN, but not to CAP and AMK. If drug susceptibility testing to KAN is not included in the standard second-line drug panel of tested drugs, the strains belonging to this lineage will have an undetected XDR phenotype. An exception to this is the strain FF359\_98 that bears a *rrs* A1401G mutation that leads to high-level KAN, AMK and CAP resistance.<sup>359</sup> EMB resistance is likely to have been acquired twice by *embB* M306V and P397T mutations. The latter mutation has been previously reported in one EMB resistant isolate.<sup>290</sup> PZA resistance was found to be acquired on multiple independent occasions through *pncA* mutations.

The Q1 subtree included two other Q1-related strains as outgroups and is characterized by a *gyrB* V340L mutation. Here, it is possible to distinguish the acquisition of INH low-level resistance by an *inhA* C-15T mutation (node B) from the acquisition of a higher INH resistance level by an *inhA* missense mutation (I194A, node C).<sup>506</sup> Some of the isolates present in the subtree were found outside the Q1 MIRU-VNTR cluster, but share more recent common ancestors with other strains in the clade, potentially indicating subsequent MIRU-VNTR divergence. The Q1 clade has, therefore, been defined as all isolates bearing the *gidB* A80P mutation characteristic of this cluster and associated with STP intermediate-level resistance previously described by some of us (Chapter 6; Perdigão *et al*, unpublished). A more linear resistance acquisition dynamic was found for this clade. EMB resistance was acquired on two

possible occasions, through an *embB* M423T (node C) and M306V (node D) mutations. RIF resistance development, leading to MDR-TB, was found to be acquired by a *rpoB* S450L mutation (node D), although a second mutation on *rpoB* (L731P) was later developed (node E). Resistance to PZA, injectable second-line drugs and FQs occurred once by mutations on *pncA* (V125G, node D), *rrs* (A1401G, node F) and *gyrA* (D94A, node G), respectively. Interestingly, isolates IHMT308\_08 and IHMT361\_08 did not show the two latter mutations in *rrs* and *gyrA* genes. Such, is inconsistent with both strains positioning in the Q1 subtree.

A further observation is that M/XDR development in the Lisboa3 subtree appeared to be accompanied by a higher genomic diversification, translated in the number of SNPs and small indels (Supplementary Tables S7.1 and S7.2). Moreover, isolates from the Lisboa3 and Q1 clades were found to bear a mean proportion of 0.73% (range: 0.2-1.8%) and 0.85% (range: 0.2-1.6%) unique SNPs, respectively, in comparison with the total SNP count of each strain. Both clades were found to share a pool of 654 (67.7-90.3%) and 626 (68.2-85.2%) common SNPs, respectively (Supplementary Figure S7.2). This intra-cluster degree of genomic uniqueness is comparable with the data reported by Niemann *et al* for the comparison of two Beijing isolates from the same outbreak clone.<sup>426</sup>

### Mutational compensation for RIF-resistance

The acquisition of compensatory mutations following resistance development has been proposed as a possible mechanism to reduce the fitness cost carried by drug resistance.<sup>262</sup> More recently, *rpoA* and *rpoC* genes were found to harbor putative RIF resistance compensatory mutations.<sup>430, 507, 508</sup> The microevolutionary analysis of Lisboa3 and Q1 clades led to the identification of two possible compensatory mutations in *rpoC* (K1152Q, node B to B1 in the Lisboa3 subtree; Supplementary Table S7.3) and *rpoB* (L731P, node D to E in the Q1 subtree; Supplementary Table S7.4) leading to RIF resistance acquisition. The *rpoA* and *rpoC* genes were screened for mutations in all isolates. On the overall 13 different non-synonymous mutations were found, of which only 6 occurred among MDR/RIF-resistance isolates (Table 7.2). The impact on protein function was inferred by computation of SIFT scores.<sup>494</sup> Only three mutations occurring in *rpoC* (Supplementary Figure S7.3) were predicted to affect protein function with SIFT scores equal to 0.00, resulting from the comparison of 189 sequences represented at each position (Table 2). The remaining mutations were predicted to be tolerated and yielded higher SIFT scores (> 0.05), resulting from the comparison of 171-189 sequences representing each position tested (Table 7.2).

We also screened the remaining RNA polymerase subunits, RpoB and RpoZ, but only eight non-synonymous mutations were identified in RpoB, concomitantly with other RIF resistance associated mutations in RpoB (Table 7.2). Five RpoB mutations (P45A, T328N, L452P, L731P and I1106T) were predicted to affect protein function after SIFT score analysis (SIFT score < 0.05) (Table 7.2).

**Table 7.2** – Candidate RIF resistance compensatory mutations found in RpoA, RpoB, and RpoC among RIF-resistant isolates with other RIF resistant associated mutations in RpoB

Protein	Mutation	SCG	No. of Isolates	SIFT Score
RpoA	E184D	2	1	0.09
RpoB	P45A	5	1	0.01
RpoB	T328N	2	1	0.03
RpoB	L452P	5	2	0.00
RpoB	V496A	6a	1	0.10
RpoB	D634G	5	1	0.49
RpoB	L731P	2, 5	13	0.00
RpoB	E812G	2	1	0.08
RpoB	I1106T	5	2	0.00
RpoC	G442C	5	1	0.00
RpoC	W484G	2	1	0.00
RpoC	D747G	4	1	0.35
RpoC	K1152Q	5	4	0.00
RpoC	S1287L	6a	1	0.23

#### Global evolution through Large Sequence Polymorphisms:

Genomes in the *M. tuberculosis* complex can downsize, through LSPs or RDs, and 89 have been previously identified.<sup>3, 78, 81, 509</sup> Across the 75 isolates, 29 (of 89) were detected as absent in at least one isolate (Supplementary Figure S7.4). The most prevalent RDs detected were RD149 (64 isolates), RD152 (45), RD174 (43), RD3 (64), RD6 (54) and RD<sup>RIO</sup> (43). As expected, all 43 strains bearing the RD174 deletion also had the RD<sup>RIO</sup> deletion.<sup>510</sup> Both deletions constitute a distinct sub-lineage within the Euro-American lineage and were detected only among SCG 5 strains.<sup>78</sup> UT205, like the Q1 and Lisboa 3 samples, had both deletions, confirming its phylogenetic proximity with these M/XDR associated strains (Supplementary Figure S7.4).

All nine isolates from the SCG 2 had the RD105 deletion characteristic of the East-Asian clade. Of these, other RD deletions were present (RD207 9 isolates, RD181 8, RD142 2). Moreover, other RD deletions associated with specific lineages were detected: RD750 (East-African-Indian

lineage, SCG 3a, 1 isolate), RD115 (Euro-American lineage, Americas-Europe sublineage, SCG 5, 8), RD183 (Euro-American lineage, Americas-Europe sublineage, SCG 3c, 1) RD193 (Euro-American lineage, Americas-Europe sublineage, SCG 4, 3), RD219 (Euro-American lineage, Americas-Europe sublineage, SCG 6a, 3) and RD761 one strain (Euro-American lineage, South Africa sublineage, SCG 5, 1 (F11 strain)) and RD724 (Euro-American lineage, Central Africa sublineage, SCG 5, 3).

No RD region was found to be absent in RGTB423 and only RD<sup>RIO</sup> deletion was detected in RGTB327. Strain RGTB423 has been found to belong to SCG 1 and PGG 1,<sup>511</sup> but in-silico PCR analysis showed that the strain had the *pks15/1* 7 bp frameshift deletion and the TbD1 deletion indicative of a modern Euro-American strain.<sup>78</sup> Nevertheless, this classification is incongruent with the SCG and PGG classification<sup>84</sup>. On the other hand, RGTB327 was found to have the RD<sup>RIO</sup> deletion only and *in silico* PCR of the *pks15/1* and TbD1 *loci* also pointed towards a modern Euro-American strain, despite the fact that deletion RD174 was not detected. Further sequencing of these two assembled strains may be required to resolve incongruences.

### Structural variability among sequenced strains

Using the sequence data, we attempted to detect structural variants (SVs), including small insertions and deletions (< 100 bp) using the Pindel program and larger variants (≥100 bp) using a combination of different methods compared with local assembly (SVMerge pipeline, Wong *et al*<sup>512</sup>). Among the group of 75 isolates, 2143 different candidate deletions (sizes 1-99 bp) and 4091 different candidate insertions (sizes 1-79 bp) were detected (Supplementary Figures S7.5 and S7.6). We have selected high quality short indels that appear to be clade associated and phylogenetically conserved among all members of the clade (Table 7.3). These variants may be clade-specific and could carry functional consequences that reflect host adaptation and selection.

**Table 7.3** - List of selected clade-defining candidate SVs, its position, size and affected ORFs. Each clade-defining candidate SV was selected based on phylogenetic congruence and presence in all members of the specified clade.

Clade	Position <sup>a</sup>	SV Type	Size (bp)	Affected ORF	Function <sup>b</sup>
KZN + CTRI_2	154139	Deletion	53	Repetitive Unit (MIRU)	
Lisboa3	3179243	Deletion	3	Rv2867c	GCN5-related N-acetyltransferase
Lisboa3	560126	Insertion	2	<i>umaA</i>	Possible mycolic acid synthase
Lisboa3 subtree (node A)	49479	Deletion	5	Rv0045c	Possible hydrolase
Lisboa3 subtree (node A)	411119	Deletion	5	<i>iniA</i>	INH inducible gene protein
Lisboa3 subtree (node A)	922934	Deletion	1	Rv0831c	Conserved protein
Lisboa3 subtree (node A)	1374937	Deletion	1	Rv1232c	Conserved protein; has a magnesium transporter domain (MgtE)
Lisboa3 subtree (node A)	2470887	Deletion	12	Rv2206	Probable conserved transmembrane protein
Lisboa3 subtree (node A)	3302325	Deletion	5	<i>fadD29</i>	Fatty-acid-AMP ligase, synthetase, synthase. Involved in biosynthesis of phenolic glycolipids
Lisboa3 subtree (node A)	3560118	Deletion	61	Intergenic	
Lisboa3(node B1)	1472921	Insertion	1	<i>rrs</i>	Ribosomal RNA 16S
Lisboa3(node B1)	1656647	Insertion	1	PE_PGRS29	PE-PGRS family protein
Lisboa3(node B1)	1918691	Insertion	2	<i>tlyA</i>	2'-O-methyltransferase
Lisboa3-A(node D1)	1769229	Insertion	1	<i>treY</i>	Maltooligosyltrehalose synthase TreY
Lisboa3-A(node D1)	2288993	Insertion	1	<i>pncA</i>	Pyrazinamidase/nicotinamidase
Q1	3006362	Deletion	1	Rv2689c	Conserved alanine and valine and glycine rich protein
Q1	3652035	Deletion	14	<i>ctpC</i>	Probable metal cation-transporting P-type ATPase C
Q1	2009775	Insertion	1	Rv1775	Conserved hypothetical protein
Q1	4408945	Insertion	8	Intergenic	
Q1 subtree (node B)	3956369	Deletion	1	Rv3520c	Possible coenzyme F420-dependent oxidoreductase
Q1 subtree (node B)	4321347	Deletion	1	Intergenic	
Q1 subtree(node B)	3230401	Insertion	7	<i>glnD</i>	Probable PII uridylyltransferase
SCG 2	99163	Deletion	9	Rv0090	Possible membrane protein
SCG 2	507029	Deletion	1	Intergenic	
SCG 2	1413130	Deletion	1	Intergenic	
SCG 2	3215623	Deletion	1	Intergenic	
SCG 2	4212833	Deletion	3	Rv3766	Hypothetical protein
SCG 2	55541	Insertion	9	<i>ponA1</i>	Penicillin-insensitive transglycosylase N-terminal domain; penicillin-sensitive transpeptidase C-terminal domain
SCG 2 + 3a	4231860	Deletion	89	Rv3785	Hypothetical protein
SCG 2 + 3a	4322040	Deletion	1	Rv3847	Hypothetical protein

Table 7.3 (cont)

Clade	Position <sup>a</sup>	SV Type	Size (bp)	Affected ORF	Function <sup>b</sup>
SCG 2 + 3a	2342650	Insertion	11	Rv2084	Hypothetical protein
SCG 4	4359136	Deletion	18	Rv3879c	ESX-1 secretion-associated protein
SCG 4	4369673	Deletion	1	<i>mycP2</i>	Probable alanine and proline rich membrane-anchored mycosin (serine protease)
SCG 4	2081450	Insertion	1	Rv1835c	Conserved hypothetical protein; has two different X-Pro dipeptidyl-peptidase domains
SCG2	3238120	Deletion	3	Rv2923c	Conserved protein; has a Osmotically inducible protein C (OsmC) domain
SCGs 2 + 3a	485811	Deletion	1	<i>pks6</i>	Probable membrane bound polyketide synthase
SCGs 3b + 3c + 4	3159999	Deletion	2	Rv2850c	Possible magnesium chelatase
SCGs 3b + 3c + 4	3929090	Deletion	9	<i>PE_PGRS53</i>	PE-PGRS family protein
SCGs 3b + 3c + 4	1612279	Insertion	6	Rv1434	Hypothetical protein
SCGs 3b + 3c + 4	3462146	Insertion	5	Rv3093c	Hypothetical oxidoreductase
SCGs 3c + 4	1573381	Deletion	1	<i>PE_PGRS25</i>	PE-PGRS family protein
SCGs 3c + 4	1951627	Deletion	1	Rv1725c	Conserved hypothetical protein; has HxIR-like helix-turn-helix domain

<sup>a</sup>Position relative to *M. tuberculosis* H37Rv and corrected from the VCF output from Pindel in order to correspond to the real insertion or deletion sites.

<sup>b</sup>Function extracted from TubercuList (<http://tuberculist.epfl.ch/>) annotation and domain information from the Pfam database (26.0) (<http://pfam.sanger.ac.uk/>)

Eight types of larger SVs were detected (Supplementary Table S7.5). Copy number gains were excluded due to a lack of robustness of the methods applied. We have found that putative SVs when detected in more than one isolate mostly present a phylogenetically incongruent distribution. The candidate structural variants found in the present study are reported (Supplementary Figure S7.7), some are identical, but only have similar breakpoints due to uneven coverage. We identified characteristic deletions for the Lisboa3 clade and the ARS6559 isolate (complete Lisboa3 subtree in Figure 4) (112 bp, position 2727803, PPE41 gene), as well as the Q1 clade strains (297 bp, position 3929891, ORF *PE\_PGRS53*). Both deletions were also validated by mapping coverage, but further laboratory confirmation is required.

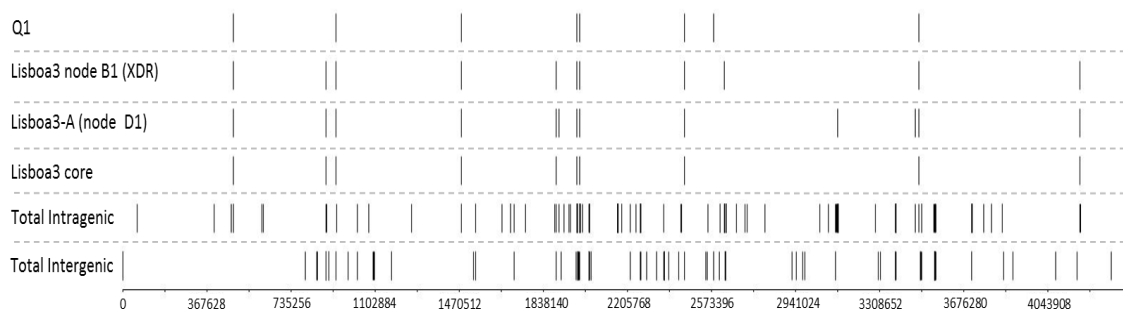
### Insertion sequence mapping and functional consequences for genomic stability

Transposition events from ISs can have a profound effect on strain physiology given the possibility of interference with gene expression by ORF knock-out or gene upregulation resulting from upstream transposition.<sup>88, 513</sup> For all strains included in the phylogenetic analysis, we attempted to map the site of all ISs annotated as mobile elements in the genome of *M. tuberculosis* H37Rv, namely IS6110. Some complex inversions were found to be predominantly transpositional events from multi-copy mobile-elements, such as IS6110. The analysis revealed the presence of IS6110, IS1081, IS1547, IS1557 and IS1558 in multiple copies, but differing in size or annotated sequence at both extremities. For this reason these ISs have been excluded from the mapping analysis.

Variability was only observed for IS1561 and IS1532 (Supplementary Figure S7.8). As expected, IS1561 was not detected in all isolates bearing the RD<sup>RIO</sup> deletion, whereas IS1532 is absent in isolates bearing the RD6 deletion found on different SCGs. For IS6110, a total of 251 candidate insertion sites have been obtained (Supplementary Figure S9), classified as of high (160), medium (18) or lesser (73) confidence. Almost half (125 (49.8%)) of the 251 ISs were observed on the positive strand. A total of 105 (41.8%) insertion sites were found to be intergenic, from which 64 (25.5%) were in the same orientation with an upstream ORF, known to exert a putative upregulatory effect. For these latter insertion sites the distance from the 3' end to the upstream ORF ranged between 0-939 bp (47 (18.7%) less than 300 bp). Thirty-three sites were found to be within PE/PPE genes, while three other insertion sites were located 18-38 bp upstream of a PPE gene.

Lisboa3 and Q1 clades were found to share 7 IS6110 sites but were differentiated by IS6110 insertions on positions 889015 (intergenic) and 4183431 (Rv3732 knock-out) for Lisboa3 and, on 2582457 (intergenic) for Q1 isolates (Figure 7.4). Moreover, we have found that strains belonging to Lisboa3-A MIRU-VNTR cluster (*rpoB* D435V clade on Figure 7.3-A) share three distinct IS6110 insertion sites on Rv1682 (position 1906425), Rv2818c (position 3125900) and Rv3096 (position 3465467). Strains from the XDR-TB Lisboa3 B1 clade (Figure 7.3-A) share a distinctive IS6110 site on the *plcC* gene (position 2628462). Although no common IS6110 site was found for the SCG 5 strains, SCG 2 strains were found to share three IS6110 sites: an intergenic site on position 888786; on Rv1754c (position 1986639); and, on Rv2820c (position 3127931). SCG 4 strains were found to also share three IS6110 sites on *mmpS1* (position 483580), PPE46 (position 3377326) and PPE47 (position 3379768). One hundred and fifty-three (60.0%) sites were found to be specific to a single isolate.





**Figure 7.4** – Genomic distribution of total mapped IS6110, intra and intergenic, and insertion sites found among Lisboa3 and Q1 isolates. Lisboa3 core and Q1 lanes depicts all insertion sites that are common to all Lisboa3 and Q1 clade isolates, respectively. Lisboa3 node B1 comprises a XDR-TB lineage shown here with an extra IS6110 copy. Lisboa3-A (node D1) are shown here to bear three additional IS6110 copies when compared with the Lisboa3 core.

Interestingly, an IS6110 insertion in the NTF locus (position 3493907) was detected in six out of the eight Beijing strains included in the analysis, which is a characteristic of the Beijing/W family (Supplementary Figure S7.9).<sup>514, 515</sup> Hence, two of the three Beijing isolates recovered in Lisbon Health Region were found to belong to the Beijing/W family. No relation with the New York City Beijing/W MDR clade was found as a second insertion in the NTF locus was not detected in any strains.<sup>514, 515</sup> Curiously, a SCG 6a strain (HPV113\_08) shared the latter insertion site with the Beijing/W strains, although only one end was detected which can be indicative of another genomic rearrangement. A SCG5 strain (HPV157\_06) was found to have an IS6110 67 bp upstream of the characteristic IS6110 insertion site of the Beijing/W family, however in a different orientation. Both insertion sites are found within the Rv3128c. This latter gene has an in-frame amber nonsense mutation in H37Rv and for this reason any functional consequence of IS6110-mediated ORF disruption is highly questionable.

Strains belonging to PGG2 were found to have a significantly lower number of IS6110 copies when compared with PGG1 strains (Kruskal-Wallis test,  $p < 0.001$ ). Given the reduced number of PGG3 strains no statistical comparison was possible to perform.

### Differential substitution ratios highlight different genomic adaptation strategies

A statistically significant  $N_s/S$  ratio was observed between Lisboa3/Q1 and Beijing strains and others, but only the Lisboa3 and Q1 result met a multiple comparison threshold

(Supplementary Table S7.7). The only significant  $T_v/T_s$  ratio occurred for differences between Lisboa3 and Q1 clusters (Q1 greater, mean difference: 0.045,  $p=0.033$ ) (Supplementary Table S7.6).

These ratios were also found to vary across the genome and across the different Clusters of Orthologous gene Groups (COGs). For each strain, we have computed the  $N_s/S$  and  $T_v/T_s$  ratio for the different genomic quadrants and for each COG. Overall quadrant  $N_s/S$  and  $T_v/T_s$  comparison, showed that  $N_s/S$  ratio varied along the chromosome such that the second quadrant had a lower  $N_s/S$  ratio when compared with the other three quadrants and that the first quadrant had the highest  $N_s/S$  mean ratio (Kruskal-Wallis,  $p<0.001$ ) (Supplementary Table S7.7). No statistical difference was observed between the third and fourth quadrant. Regarding the  $T_v/T_s$  ratio, an approximately inverse situation was found as no statistical difference was observed between the first, third and fourth quadrants. The second quadrant showed however, a significantly higher  $T_v/T_s$  ratio than the three other quadrants (Kruskal-Wallis,  $p<0.001$ ) (Supplementary Table S7.7).

When these results were stratified by genetic clade, it was found that in the first quadrant the Beijing strains showed a statistically lower  $N_s/S$  ratio upon comparison with Q1 and other non-clustered (NC) strains, but not Lisboa3 (Supplementary Table S7.8). No statistical difference was found in this quadrant for  $T_v/T_s$  ratio. In the second quadrant, Lisboa3 strains showed a statistically significant reduced  $N_s/S$  ratio compared with the other three groups of strains, while Beijing strains presented a higher  $N_s/S$  ratio than the remaining groups (Supplementary Table S7.8). Inversely, the  $T_v/T_s$  ratio on the second quadrant was significantly higher for Beijing strains when compared to Q1 and other NC strains, but not to Lisboa3 strains (Supplementary Table S7.8). The analysis of the third quadrant showed no statistical difference for  $N_s/S$  ratio while Beijing strains showed a higher  $T_v/T_s$  ratio on comparison with Lisboa3 and other NC strains, but not Q1 strains. Lisboa3 strains showed a reduced  $T_v/T_s$  ratio on this latter quadrant when compared to all other groups. In the fourth quadrant, only a statistical difference was observed for a Q1 reduced  $N_s/S$  ratio when comparing with the other strain groups and no significant difference was observed for the  $T_v/T_s$  ratio (Supplementary Table S7.8).

These results show that the  $N_s/S$  and  $T_v/T_s$  ratio measures appear to vary on a strain and chromosome region dependent mode. Data stratification by isolate and quadrant showed that the  $T_v/T_s$  ratio was found to correlate negatively with the  $N_s/S$  ratio (Pearson,  $p<0.001$ ).

Correlation between overall isolate  $N_s/S$  and  $T_v/T_s$  ratio was also attempted but no correlation was found (Pearson,  $p=0.433$ ).

The comparison of the  $N_s/S$  and  $T_v/T_s$  ratios across the different COGs also yielded strain dependent results. On comparison with the other three strain groups: Lisboa3 strains showed higher  $N_s/S$  ratios on COG groups D (Cell Cycle Control, Mitosis and Meiosis) and P (Inorganic Ion Transport); Q1 strains showed higher  $N_s/S$  ratios on COG group V (Defense Mechanisms); and, Beijing strains showed higher  $N_s/S$  ratios on COG groups F (Nucleotide Transport and Metabolism), K (Transcription), N (Cell Motility), O (Post translation Modification, Protein turnover and Chaperones) and Q (Secondary Metabolites Biosynthesis, Transport and Catabolism) (Supplementary Table S7.9). Regarding the  $T_v/T_s$  ratio no significant difference was observed for Lisboa3 strains, but higher ratios were observed for Q1 strains in COG groups J (Translation), L (Replication, Recombination and Repair), M (Cell Wall, Membrane Biogenesis) and, for Beijing strains in COG group C (Energy Production and Conversion) (Supplementary Table S7.10).

These results support the notion of a differential mode of evolution and adaptation to the human host by accumulation/selection of a higher degree of non-synonymous mutations at genes belonging to specific functional categories.

According to recent work by Namouchi *et al.*,<sup>77</sup> the  $N_s/S$  ratio varied along the phylogenetic tree, such that terminal branches had a higher  $N_s/S$  ratio than inner branches. We have computed the  $N_s/S$  and  $T_v/T_s$  ratio for the inner nodes assigned in the subtrees in Figure 3 and compared with the respective ratios calculated for the tips of the subtrees. Contrary to the data of Namouchi *et al.*,<sup>77</sup> we have verified that both subtrees had  $\approx 6\%$  and  $\approx 12\%$  lower  $N_s/S$  ratios at the tips of Lisboa3 and Q1 subtrees, respectively, when compared with the inner nodes of the tree (Independent t-test,  $p<0.001$ ). For the  $T_v/T_s$  ratio, the opposite was found: higher  $T_v/T_s$  ratios were observed at the tips in comparison with the inner nodes (Mann-Whitney test,  $p<0.001$ ).

## 7.4 DISCUSSION

For at least two decades the Lisbon Health Region in Portugal has been characterized by a high-level of drug resistance, at first MDR-TB, and later XDR-TB, mainly caused by a particular group of strains: the Lisboa family. Presently, this drug resistance is due almost in its entirety

to an endemic circulation of the Q1 and Lisboa3 phylogenetic clades. Present data from 24-*loci* (not 12-*loci*) MIRU-VNTR allowed the subdivision of the Lisbon3 cluster in two other clusters herein designated as Lisboa3-A and -B. This data suggests two independent outbreaks, over the years, dated back to 90s when the discrimination of Lisboa strains was identified by distinct *rpoB* mutations.<sup>405</sup> The Q1 spoligotyping data has revealed that this cluster is in fact intimately related with the B cluster identified in 90s outbreak (unpublished data). Phylogenetic analysis based on previously published sets of SNPs revealed that Lisboa3 and Q1 strains formed distinct monophyletic evolutionary clades within the SCG 5 and PGG 2.<sup>52, 66</sup> Interestingly, *M. tuberculosis* F11 and the XDR-TB associated KZN strains, both originating from South Africa also belong to SCG 5. Nevertheless a clear distinction is highlighted in the proposed phylogeny. This distinctiveness is also reflected by the RD comparison, but Lisboa3, Q1 and KZN strains appear to have an incongruent phylogeographic association using the RD typing. All these strains belong to the Euro-American lineage according to the RD classification proposed by Gagneux *et al.*<sup>78</sup> However, the KZN strains included in the analysis showed to be positive for RD115, associated with an Americas/Europe sublineage, despite the fact that these strains are a major public health concern in South Africa, namely, the XDR-TB outbreak in KwaZulu Natal.<sup>516, 517</sup> The Lisboa3 and Q1 strains were on the other hand positive for RD174, associated with a West-African sublineage, but constitute a major public health concern in Europe. Present knowledge recognizes that RD174 is also associated with RD<sup>RIO</sup>, an LSP that has initially been discovered in Rio de Janeiro, Brazil but was later found to be widespread. Historic ties connect Portugal, Brazil and West African Countries and a possible ancestor for these two clades might lie in Africa, more specifically on Portuguese Speaking African Countries. These phylogeographic incongruences are consistent with human migratory events out from, and back into, the African continent.<sup>2</sup>

Another question still seems pertinent as to which selective advantages do these two clades possess allowing such high prevalence in this setting especially since other strains, *e.g.* pre-XDR-TB Beijing strains, also do circulate but at an apparent lesser prevalence? TB caused by RD<sup>RIO</sup> strains has shown to be associated with weight loss, hemoptysis, higher bacillary loads and progression to cavitory disease.<sup>509, 518</sup> This deletion encompasses several PPE genes that have shown to be a potential source of immune variation (reviewed in Akhter *et al.*<sup>519</sup> and Mukhopadhyay *et al.*<sup>520</sup>) and hence, may constitute a pathogenic adaptation strategy to immune evasion. Higher bacillary loads are associated with a higher secondary case rate<sup>521-523</sup> and if in fact the absence of these genes truly plays an important role towards an increased virulence, or even transmissibility, it may be a factor that has contributed to the high

prevalence of RD<sup>RIO</sup> strains in this setting simultaneously contributing to the emergence and spread of M/XDR-TB strains.

The finding that a 112 bp deletion is present among Lisboa3 clade strains, with a more restricted distribution than RD<sup>RIO</sup>, affecting gene PPE41 might also provide additional clues and contribute to a higher virulence or transmissibility. PPE41 has been previously described as having an immunodominant nature and shown to activate a CD4<sup>+</sup> and CD8<sup>+</sup> mediated T cell response leading to an enhanced IFN- $\gamma$  response as well as induce a strong humoral response.<sup>524, 525</sup> The deletion found might constitute a means of immune evasion and constitute a selective advantage over other circulating strains. More specifically, a stronger humoral response to PPE41 was found among extra-pulmonary TB patients.<sup>525</sup> The selective advantage provided by this deletion might therefore also be related with the fact that Lisboa strains were first identified among HIV-infected patients, which is associated with an increase in extra-pulmonary TB.

The use of SNPs as molecular markers has contributed to an improved understanding of the evolutionary history of the *M. tuberculosis* complex. In the present study, given the availability of genomewide SNP data, a SNP-based phylogeny was deduced from the genomic data and, overall, the proposed phylogeny appears to be consistent with other SNP-based phylogenies although as already pointed out: SCG 3 does not exist as a monophyletic lineage but instead as a paraphyletic one. The original report by Filliol *et al*<sup>64</sup> proposed a minimum number of sixteen SNPs that allowed assignment of any strain to an SCG but not to its subgroupings. A later erratum showed that SCG 3a belonged in fact to PGG1 while SCG 3b and 3c belonged to PGG2 as confirmed by our results. Alland *et al* proposed instead a set of nine SNPs that allowed strain assignment to any SCG and each subgroup.<sup>66</sup>

The phylogeny constructed in the present study contributes nevertheless to demonstrate the uniqueness of Lisboa3 and Q1 strains in a global context and will comprise a future framework for genome-wide association studies (GWAS).

The phylogeny proposed also enabled a microevolutionary perspective on the path towards MDR and XDR. As expected, in the Lisboa3 and Q1 clades, INH resistance was found to be mediated by double *inhA* promoter/structural mutations, recently described by some of us to contribute to INH high-level resistance.<sup>506</sup> The acquisition of *inhA* C-15T mutation was found to have occurred independently in both lineages, and in Q1 cluster it was possible to determine that C-15T mutation was acquired at a first stage of INH high-level resistance development. In Lisboa3 it was not possible to determine which mutation appeared in the first place since no

Lisboa3 isolate with a single *inhA* mutation was found. Recent work by Fenner *et al* suggested that *inhA* promoter mutations, more specifically C-15T mutation, might be associated with Lineage 1 (Indo-Oceanic/SCG 1).<sup>84, 526</sup> Nevertheless, another earlier study from Brimacombe *et al* showed that SCG 1 and 5 had all the mutations of interest towards INH resistance.<sup>527</sup> In our view, the fact that INH resistance in both Lisboa3 and Q1 clades is associated with *inhA* mutations, instead of the more usual KatG mutations, is possibly related with selective pressures exerted by the drug regimen itself.

The analysis of Lisboa3 subtree has further highlighted the M/XDR evolutive process in this clade. We have recently proposed an evolutionary path regarding drug resistance acquisition dynamics based on the acquisition of an *eis* promoter mutation as the first-step from MDR to XDR.<sup>434</sup> However the SNP phylogeny proposed is consistent with a twice and independent acquisition of an *eis* promoter mutation. Given this phylogeny it is not possible to establish any order of mutation acquisition. Nonetheless, instead of a single event, our analysis supports multiple development of XDR-TB in the same phylogenetic clade. Two different transmission chains involving strains with the RpoB S450L, instead of one, are also more likely since it is proposed that this mutation has also been acquired twice and independently.<sup>405</sup>

One striking phylogenetic incongruence was found in two Q1 strains that lacked both second-line injectable drug and FQ genetic resistance determinants and at the same time sharing a recent common ancestor resistant to these two classes of drugs. These two strains were genotypically and phenotypically susceptible to AMK, CAP and any of the FQs tested. Two explanations may be considered: a phylogenetic misplacement, although the branches had a good statistical support or, these strains may descend from a reverter ancestor. Although theoretically possible, events such as these may be extremely rare. Only one report has documented an in-patient reversion of an isogenic strain from INH resistant to susceptible.<sup>528</sup>

The acquisition of further mutations in *rpoA*, *rpoB* or *rpoC* genes following RIF resistance development was recently demonstrated, using *Salmonella* as a model organism, to have an important role in fitness compensation, leading to a reduction in the doubling-time to values closer to the wild-type.<sup>529</sup> In our microevolutionary analysis we have detected a RpoC mutation (K1152Q) occurring in the same branch as a RpoB S450L (equivalent to S531L in RpoB *E. coli* numbering). It is the first description of a putative compensatory mutation within the Lisboa3 clade, contributing to the success of one of its sub-lineages through the amelioration of the resistance fitness cost.<sup>262</sup> RIF compensatory evolution has been the subject of two recent studies that showed a high prevalence of *rpoA* and *rpoC* mutations mapped to the

RpoA-RpoC interaction region.<sup>507, 508, 530</sup> The *rpoC* mutation described in a Lisboa3 sub-lineage does not fall in this region, nor was it described in these studies.<sup>507, 508</sup> Nevertheless, two other putative compensatory mutations mapping to the RpoA-RpoC interaction region were found in other isolates not belonging to the Lisboa3 or Q1 clades (Supplementary Figure S7.3). The putative role of these two latter mutations is only substantiated by the bioinformatic analysis of residue conservation. However, the putative compensatory role of the Lisboa3 K1152Q RpoC is further substantiated by their co-occurrence in the same branch as the RIF resistance determining mutation in *rpoB*. Furthermore, none of these putative compensatory mutations was previously described and may constitute novel polymorphisms associated with molecular RIF resistance compensation.<sup>430, 507, 508</sup>

RpoB mutational analysis also allowed the identification of five putative compensatory mutations, of which one (L731P) was found to be acquired in the Q1 clade following RIF resistance acquisition through another *rpoB* mutation. This latter mutation was found to be homoplasic as it was also detected in different SCGs, which also points towards the usefulness of this mutation to counteract fitness costs imposed by the acquisition of other RIF resistance associated mutations. Mutations outside the RIF resistance determining region on *rpoB* gene have been described previously on RIF-resistant isolates with no mutations on this region.<sup>249, 261</sup> The mutations herein described as putatively compensatory were only considered as such if a mutation in the RRDR was already present providing further support for the compensatory role of the former.

The role of compensatory mutations in other *loci* and associated with compensation to resistance to other drugs than RIF have been identified and studied, namely, mutations on *ahpC* for INH or on *rrs* for second-line drug aminoglycosides.<sup>531-533</sup> Nevertheless, no compensatory mutations were identified in these genes (data not shown).

Insertion site mapping revealed a high genomic stability of insertion sequences other than IS6110. In fact, we have verified that only deletion events were responsible for variability regarding presence/absence of an insertion sequence other than IS6110. On the other hand, as demonstrated in this study IS6110 is a highly polymorphic marker, probably due to its rapid transposition rate.<sup>534, 535</sup>

The finding that 65.5% of the IS6110 insertion sites mapped were located intragenically is in line with previous reports.<sup>536</sup> Considering that ~91% of *M. tuberculosis* genome is composed by coding regions,<sup>62</sup> it highlights the deleterious effects of transposition into certain genes essential to viability or to the successful completion of the pathogen's infectious cycle. PGG1

strains, including the Beijing strains, were found to bear a higher number of IS6110 copies than PGG2 strains. IS6110 copy number is presumed to be under negative selection,<sup>537</sup> however, in certain circumstances, it is the insertion site *per se* that might provide a selective advantage and not the copy number.

Considering the data obtained in this study, IS6110 is unarguably the species' most important mobile element when considering transposition impact on genomic integrity. IS6110 appears to have an important role in genomic re-shaping towards adaptation either through localized disruption of putative antigenic targets (*e.g.* PPE/PE genes) or through its mobile promoter activity located in the IS6110 3' end, capable of inducing transcription or upregulating the expression of downstream genes under stressful conditions.<sup>90</sup> We have found a considerable number of insertion sites to be within PPE genes and a more reduced number of sites to be upstream of PPE genes. PPE genes appear to have been positively selected in pathogenic mycobacteria, have important immune and antigenic potential, and some can induce a shift towards a Th2-type response.<sup>519, 520</sup> Not only the IS6110-mediated disruption of PPE genes might constitute a mean of immune evasion but it is also conceivable that upregulation of specific PPE genes might affect the Th1/Th2 response balance.

Remarkably, Lisboa3 and Q1 did not show any IS6110-mediated disruption of a PPE ORF, nor did we found any IS6110 upstream of a PPE gene. This fact perhaps demonstrates a different mode of evolution and host adaptation that does not require PPE gene modulation through IS6110 transposition.

Nevertheless, the maximum distance between an IS6110 and a downstream gene so that this 3' promoter can exert its influence on gene expression is unknown. The results reported by Safi *et al*<sup>90</sup> demonstrate that an IS6110 in *M. tuberculosis* 210 located 297 bp upstream of Rv1468c was associated with a threefold increase in transcription upon macrophage infection. Our results show that 15% of the mapped sites are located upstream of an ORF in proper orientation and at a distance of less than 300 bp which, at the light of present knowledge, fulfills the necessary assumptions to exert a putative upregulatory effect on those ORFs. Also considering the diversity of ORFs interrupted by IS6110 copies, gene knock out studies and assessment of downstream gene expression are necessary if a functional role for specific transposition events is to be established.

Spoligotyping lineage association with specific IS6110 sites has already been demonstrated, highlighting the phylogenetic informativeness of this marker.<sup>538</sup> Our results also support an association of specific IS6110 sites with strain lineage at both global and local levels.



Notably, the comparison of the distribution of SNPs by COG showed that  $N_s/S$  ratios vary through COG in a lineage-dependent manner. Although, Lisboa3 and Q1 isolates might be overrepresented in the analysis due to the high prevalence in the community, we have shown that Lisboa3 and Q1 present statistically different  $N_s/S$  ratios from the remaining isolates. We propose that differences in  $N_s/S$  COG might highlight different evolution strategies selected during host-pathogen interaction and adaptation.

Moreover, an overall higher  $N_s/S$  ratio was observed for the first quadrant and an overall lower  $N_s/S$  ratio was observed for the second quadrant revealing heterogeneous  $N_s/S$  ratios negatively correlated with the  $T_v/T_s$  ratio. The biochemical nature behind this  $T_v/T_s$  ratio heterogeneity requires further studies as it may be driving localized higher non-synonymous mutation rates with functional impact on strain evolution. The precise genes affected by non-synonymous mutations within these COG categories merit further studies as each COG includes a considerable number of genes, that mutated might enhance the transmissibility or drug resistance, and should be analyzed in a systems biology perspective using *in silico* models.<sup>539-541</sup>

The finding that terminal branches of the subtrees analyzed had lower  $N_s/S$  ratios than the inner branches was contrary to the findings of Namouchi *et al.*<sup>77</sup> Namouchi *et al* suggested that non-synonymous changes might be purged by natural selection yielding higher  $N_s/S$  ratios. However, an opposite view is also possible: non-synonymous mutations are favored by natural selection yielding the same higher  $N_s/S$  ratio, especially as a mean of adaptation in an organism devoid, or with low, horizontal gene transfer such as *M. tuberculosis*.<sup>72, 76</sup> From our data, we can say that non-synonymous mutations may be favored in the inner branches of both subtrees as a mean to develop and adapt to drug resistance, yielding a higher  $N_s/S$  ratio that is consistent with a reduced purifying selection.<sup>2</sup> Nevertheless, the differences between the results from both studies might lie in the fact that the  $N_s/S$  ratio analysis herein presented was performed for two sets of strains that are in a much more closer time frame in order to understand microevolution within two clades.

In the present study we have shown that in Lisbon, Portugal, where the MDR-TB situation had already escalated to a XDR-TB situation, it is mainly caused by transmission of two unique phylogenetic clades. We have previously shown that XDR-TB was already a reality in Portugal during the 1990s but noteworthy, the data from the present study clearly shows that these strains belong to the same phylogenetic Lisboa3 M/XDR sublineages that are still presently in

circulation.<sup>434</sup> The uniqueness of these strains was revealed by a distinct phylogenetic placement within SCG 5.

The Lisboa3 clade belongs to a much broader group of strains that usually share at least 90% of MIRU-VNTR pattern similarity: the Lisboa family.<sup>26</sup> One of the future goals is to better understand the populational structure of this family of strains, from which the Lisboa3 clade has differentiated, as strains belonging to this family have previously shown the potential to evolve to MDR-TB.<sup>24</sup>

In conclusion, the molecular diversity described in the present study greatly contributes to the body of knowledge concerning *M. tuberculosis* diversity and supports the notion of higher genomic diversity than the one usually associated with *M. tuberculosis*, mostly acquired through genome downsizing and non-synonymous SNPs. The present study also stresses the need of further genomic studies in order to contribute to a *M. tuberculosis* genome-wide evolutive scenario, representative of different settings. This, together with clinical data, will ultimately enable GWAS with a positive impact in TB management.

## 7.5 MATERIALS AND METHODS

### Isolates and genetic data

The study consists of 56 *M. tuberculosis* clinical isolates (source: 55 Lisbon, 1 Oporto) recovered from laboratories and hospital units across Lisbon Health region. All isolates underwent drug susceptibility testing for INH, RIF, STP, EMB and PZA and second-line drugs using standard methods (see Perdigao *et al* <sup>24</sup>). For MDR-TB strains, drug susceptibility testing was also performed for ETH, CAP, AMK and OFX. DNA extraction was performed from culture growth on Lowenstein-Jensen medium slants using the cetyl trimethylammonium bromide methodology.<sup>445</sup> The DNA was used in genotyping by the 24-*loci* MIRU-VNTR method (see previous work, Supply *et al* <sup>56</sup>). Extracted DNA was also subjected to whole-genome (101bp paired end) sequencing at the KAUST genomics facility using the Illumina HiSeq 2000 platform (500bp insert size). We also complemented this data using sequences in the public domain (F11, CDC1551, KZN1435, KZN4207, KZN605, KZN\_R506, CM000789.2; KZN\_V2475, UT205 RGTB327, RGTB423, CCDC5180, CCDC5079, CTRI-2, BTB05\_552 BTB05\_559, S96\_129, HN878, R1207, and X122 (all from the NCBI database).

## Genomic variant detection

The raw Illumina sequencing data was aligned to the H37Rv reference genome using the Burrows-Wheeler Alignment Tool v.0.6.1.<sup>542</sup> SNPs and small indels (<30 bp) were called using SAMtools software (v0.1.18) to the Variant Call Format (VCF).<sup>543, 544</sup> Other small indels (<100 bp) were detected using the software Pindel.<sup>545</sup> Only variants supported by at least ten sequence reads were considered. Detection of larger structural variants was performed using the SVMerge v1.2 pipeline combining Pindel v0.2.4t, BreakDancer v1.1 Cpp package and, SECluster analysis outputs.<sup>512, 546</sup> Structural variant detection was done for each isolate alone and validation was achieved using comparison with local *de novo* assembly using Velvet.<sup>547</sup> Loci reported to be associated with regional differences were identified using the alignments and coverage.<sup>3, 78, 81</sup>

For IS mapping, reads containing specific oligonucleotide sequence of both 5' and 3' extremities (listed in Supplementary Table S7.11) were extracted and, flanking genomic regions of interest concatenated in FASTA format producing one file for each extremity for each strain. Local BLAST analysis (standalone NCBI BLAST v.2.2.27+) was carried out for each file against *M. tuberculosis* H37Rv reference genome, minimum supporting read depth used to as a quality filter (10 for isolates with >500 fold coverage, 2 for the remaining). For IS6110 BLAST hits, a mapping quality classification scheme was established consisting in high confidence, medium confidence and lesser confidence sites. Paired sites corresponding to mapping of both 5' and 3' ends in all isolates on which it occurred were classified as high confidence sites. Paired insertion sites for which both ends were mapped in at least 50% of the isolates on which they were found to occur were considered of medium confidence. Insertion sites in which only one end of the IS6110 was mapped were considered of lesser confidence. Furthermore, insertion site hits mapped to *M. tuberculosis* H37Rv were excluded to avoid repetitive mapping.

## Other bioinformatics

The genomic data of publicly available *M. tuberculosis* strains (format FASTA) were included in the analysis through conversion to FASTQ format reads using the program dwgsim v.0.1.10, and mapped and analyzed as described above. When necessary, DNA sequence alignment was performed using the CLC Sequence Viewer v7.6.1 (CLC bio™, Aarhus N, Denmark) and visualized in BioEdit v7.1.3.0 (T. Hall).

A MIRU-VNTR-based dendrogram was constructed in the public MIRU-VNTR*plus* database using the  $D_{sw}$  measure of genetic distance for tandem repeat *loci* and the UPGMA clustering method.<sup>548</sup> A phylogenetic tree based on SNPs was constructed using Seaview 4.3.5 using the Maximum Likelihood method.<sup>549</sup> The analysis involved 76 nucleotide sequences with a total of 11271 sites in the final dataset. Tree topology was tested using the most recent approximate Likelihood Ratio Test (aLRT) as an alternative to bootstrap.

Putative impact of selected compensatory mutations on protein function was assessed through the use of SIFT scores (available at <http://sift.jcvi.org/>) computed from the query alignment against UniRef90 database hits (with less than 90% identity, with a median sequence conservation equal to 3.00).<sup>494</sup>

Any statistical analysis was conducted using the SPSS software.

## **7.6 ACKNOWLEDGEMENTS**

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## **7.7 DISCLOSURE DECLARATION**

The authors have no competing interests to declare.

## **CHAPTER 8**

### **General Discussion**



## 8.1 GENERAL DISCUSSION AND FUTURE PERSPECTIVES

MDR-TB imposes a heavy burden on public health systems and constitutes from a public health perspective a matter of great concern. TB incidence has been declining in Portugal over the last decades, which *per se* is a good indicator.<sup>21, 23</sup> Nevertheless it is MDR-TB and its even more difficult to treat form - XDR-TB, that raises more questions on its management and control. In fact, attending to the global tendency on the number of new cases of MDR-TB and XDR-TB reported by PHA no specific trend can be clearly observed but rather oscillating values.<sup>23</sup>

In the present work we have mainly addressed the study of MDR-TB in the Lisbon Health Region along with its molecular determinants at the epidemiological and resistance levels. One limitation of this work is that all sets of isolates are fundamentally convenience samples obtained through collaborations with the Portuguese National Institute of Health and other laboratories in the region. Despite this, the considerable number of clinical isolates studied, together with the fact that all M/XDR-TB sample sizes are higher than the number of new M/XDR-TB cases reported nationwide should provide some degree of representativity.

This work therefore comprises several studies that were aimed at describing epidemiological aspects of MDR-TB in Lisbon through molecular biology methods and simultaneously, contribute to the global knowledge on *M. tuberculosis* genetic diversity and adaptation to chemotherapy regimens. We started by addressing MDR-TB (Chapter 2) in which we describe the clonality and first-line drug resistance associated mutations in circulating MDR *M. tuberculosis* isolates. We uncovered the main mutations associated with first-line drug resistance. It became noteworthy the high prevalence of *inhA* promoter mutations in INH resistant isolates and not, as usually is expected, a high prevalence of *katG* mutations, namely on the 315 codon. Mutations in the promoter region of *inhA* gene appear to have evolved independently, at least twice, for Q1 and Lisboa3 isolates as demonstrated in Chapter 7. Muller *et al* have described these mutations as a gateway for XDR-TB in South Africa due to its statistical association with XDR-TB.<sup>225</sup> Nevertheless, in Lisbon the INH resistance level is higher than the one anticipated by these authors, due to two different and recently described mutations co-occurring in the *inhA* ORF.<sup>550</sup> For Q1 isolates it was shown in Chapter 7 by microevolutionary analysis that the *inhA* promoter mutation was acquired in the first place while the InhA structural mutation was acquired in a second stage.

Additional mutations are described in Chapter 2 as well as the clonality assessment, which is further discussed below, showing a major prevalence of Lisboa3 strains. However, and perhaps

the most striking observation was in fact the high XDR-TB rate found in the analyzed sample (56%). This high XDR-TB rate motivated the studies on the molecular determinants of resistance to second-line drugs, in Chapter 3, and on the six-year laboratory perspective on the isolation of M/XDR-TB *M. tuberculosis* strains, in Chapter 4.

In Chapter 3, the analysis of 26 XDR-TB clinical isolates showed a high clustering degree with the 26 isolates belonging to one of either two 12-*loci* MIRU-VNTR clusters: Lisboa3 and Q1. The mutational analysis of *gyrA*, *rrs* and *tlyA* genes revealed that cluster-associated mutations were found in all three genes. Worth mentioning, a 2 bp insertion in *tlyA* gene is proposed to be responsible for acquired CAP resistance in Lisboa3 cluster isolates that do not show any classical *rrs* mutation. This finding has strong implications for the implementation in Lisbon of the only commercial NAAT able to screen for second-line drug resistance associated mutations (GenoType™ MTBDRsl, Hain Lifescience). The implementation of this test would leave undetected about 58% of the CAP resistant isolates. Moreover, no molecular markers for AMK/KAN resistance were found for these Lisboa3 cluster isolates.

The high rates of XDR-TB and MDR-TB were further investigated by analyzing laboratory records of clinical isolates recovered over a six-year period. In Chapter 4 we have shown that the high MDR-TB and XDR-TB were, on the overall, maintained over the period studied. These observations indicate that the M/XDR-TB high rates are not due to a punctual outbreak but to a continued circulation of M/XDR-TB strains mostly belonging to the previously identified clusters Lisboa3 and Q1. Moreover, an increase in the fraction of MDR-TB resistant to all five first-line drugs was observed. The increase in this fraction over isolates that are resistant to a lesser number of first-line drugs might illustrate a situation in which standardized treatment results in selection of strains with additional resistances leading to resistance amplification. The repetition of this phenomenon allied with transmission can result in more cases of primary drug resistant TB.

It is also important to address here the differences in MIRU-VNTR profiles between the same clusters (Chapters 2, 4, 6 and 7). MIRU-VNTR reproducibility may be affected by difficulties in sizing and allele assignment. The initial report on MIRU-VNTR sizing by capillary electrophoresis used the GeneScan 2500 ROX (Applied Biosystems™) molecular weight marker which is adequate for non-denaturing systems. Later, the MIRU-VNTR technical guide recommended the use of MapMarker™ 1000 ROX.<sup>551</sup> Our experience with both markers show that the use of MapMarker™ 1000 ROX results in a much more accurate peak sizing than with GeneScan™ 2500 ROX. Subsequently, MIRU-VNTR typing and calibration kits have been



developed and commercialized by Genoscreen™ for both improved multiplex amplification and sizing, respectively. Nevertheless, the price of these kits for routine use in molecular epidemiological surveys is still prohibitive in many settings. This latter kit recommends using GeneScan™ 1200 LIZ as a molecular weight marker; however, we prefer MapMarker™ 1000 ROX as electrophoresis visual inspection of raw data is possible given the regular band pattern of the latter. MIRU-VNTR profiles of Lisboa3 and Q1 in Chapter 7 should now be considered the correct ones as sizing was carried out using a custom MapMarker™ molecular weight marker derived from MapMarker™ 1000 ROX but, with additional bands up to 1200 bp. Additionally, a calibration step was also performed through the sizing of MIRU-VNTR *loci* allelic ladders from the Genoscreen™ MIRU-VNTR Calibration Kit with the custom MapMarker™ 1200 ROX.

MIRU-VNTR typing started to be implemented in our laboratory in 2004 for typing of strains recovered in previous years. Peak sizing difficulties that affect the inter- and intralaboratory reproducibility of the technique hamper direct comparison with strains genotyped in the following years (between 2004-2006).<sup>552</sup> For this reason, genotype comparison in Chapter 4 was only made for strains recovered between 2004-2006. Moreover, looking back to our results with the strains from 2003 (Chapter 2) we would now expect a higher clustering rate taking in account the distribution of *pncA* gene mutations and knowledge from microevolution analysis (Chapter 7). Reconstruction of a calibrated MIRU-VNTR allele sizing table is recommended for implementation of MIRU-VNTR typing (Chapter 7).

Given the fact that all the studies in Chapters 2-6 involved genotyping with 12-*loci* MIRU-VNTR one of the main objectives was to progress towards the implementation of a technique with a more powerful discriminative power. To accomplish this, genotyping using additional MIRU-VNTR *loci* was successfully implemented up to the 24-*loci* MIRU-VNTR typing method. Therefore, in Chapter 7 lies also the first proposal for 24-*loci* MIRU-VNTR profiles of Lisboa3 and Q1 strains. The inclusion of further *loci* allowed a better discrimination of these clusters with special importance for the Lisboa3 cluster. It was possible, by using 24 *loci* for MIRU typing, to identify two clusters: Lisboa3-A and -B, of which only Lisboa3-B was found to be associated with XDR-TB. Using a lesser number of *loci* has nevertheless the advantage of identifying more deep phylogenetically related isolates that otherwise may appear as outgroups and not as strains that have undergone MIRU allelic diversification. The ability to now genotype *M. tuberculosis* clinical isolates by the 24-*loci* MIRU-VNTR method will also be used in upcoming studies that will support new perspectives on the populational genetic structure of *M. tuberculosis* in the region (unpublished data). The use of specific models to

analyze this type of multilocus data will enable the definition of the number of populations present within a setting that, if cross-referenced with the patient demographic data, might allow the development of programmatic intervention strategies at the community level.

Since the description of mutations associated with second-line drug resistance that the cross resistance between the second-line injectable drugs has raised questions concerning the mechanisms behind the observed resistance patterns. A study published by Zaunbrecher *et al*, in 2009, revealed that overexpression of the *eis* gene, coding for an acetyltransferase, was responsible for resistance acquisition towards KAN but not AMK.<sup>372</sup> This finding *per se* is interesting in the way that it constitutes a novel drug resistance mechanism in *M. tuberculosis*: overexpression of a drug-modifying enzyme. In Chapter 5 we found that in an extended set of isolates, including those analyzed in Chapter 3, 19 had an *eis* promoter mutation and, proposed a model of stepwise resistance acquisition in Lisboa3 strains. However, the microevolution analysis (Chapter 7) showed that *eis* G-10A mutation is proposed to have been acquired independently in Lisboa3 clade evidencing multiple resistance development and convergent evolution within a cluster. This more recent phylogenetic analysis based on genomewide SNPs is consistent with multiple emergence of XDR.

KAN resistance may therefore be explained by *eis* promoter mutations. It is, in principle, more likely that this *eis* promoter mutation has been selected by a KAN-containing regimen. Although KAN is said not to be used to treat TB in Portugal as it is not available, the fact that two isolates recovered in the 90s already had the profile of XDR-TB may be consistent with KAN use in TB treatment regimens in the past. It also highlights the fact that XDR-TB transmission was already ongoing in Portugal for at least nine years before the very existence of a XDR-TB definition.<sup>432</sup>

Eis is a known virulence factor that is associated with cellular surface and released to the medium.<sup>489, 553</sup> Wei *et al* have shown that *M. tuberculosis* Eis overexpression in *M. smegmatis* enhances intracellular survival in macrophages while Wu *et al* showed that *eis*-deficient *M. tuberculosis* strains show decreased growth on MonoMac6 cells.<sup>554, 555</sup> Furthermore, it has been suggested that differences in *eis* gene expression levels might translate in different growth rates on MonoMac6 cells by comparison of growth rates and *eis* expression levels in both *M. tuberculosis* H37Rv and TB294 (strain 210 isolate).<sup>555</sup> Upregulation of *eis* gene as a result of selection for KAN resistance may, nevertheless, carry out an increase in virulence. If such is to be confirmed, the use of KAN in treatment regimens should also be revised as it may select for strains with enhanced virulence. Also, this event would contribute to an increased

virulence by certain strains within the Lisboa3 clade and help to explain the high prevalence of these strains in Lisbon Health Region.<sup>24, 482</sup>

One other question remains: despite the fact that *eis* gene product is reported to acetylate KAN with a much higher affinity than AMK, does this lesser affinity result in an AMK MIC increase of clinical significance? Only a two-fold increase in the AMK MIC was observed for one strain, while the other strain did not show any change when compared with *M. tuberculosis* H37Rv. Increase of *eis* gene expression has been shown to be triggered by the upregulation of the principal *M. tuberculosis* sigma factor, SigA, upon macrophage infection.<sup>555, 556</sup> One hypothesis would be that this *in vivo* activation combined with a *eis* promoter hypermorphic mutation would result in a higher resistance level than the one that can be determined *in vitro* using standard DST methods. However, *eis* gene expression from a multicopy plasmid in *M. smegmatis* appears to have no effect on AMK MIC.<sup>554</sup> It will be of future interest to determine the AMK resistance level associated with *eis* promoter mutations using a more sensitive method at lower concentrations, such as the qDST. The study of the survival rates of these strains upon macrophage infection, under different AMK concentrations, might also provide additional data relevant to whether or not use AMK in drug regimens designed to treat infections caused by strains with *eis* promoter mutations.

An additional and relevant implication for regimen design concerns *tlyA* mutations as a major mechanism of CAP resistance in the region. Based on the known possible resistance mechanisms a hierarchy for usage of AMK/KAN or CAP can be suggested. As demonstrated by our data, one of the potential mechanisms leading to CAP resistance are *tlyA* mutations, which do not confer resistance to AMK/KAN. Contrary to the recent WHO guidelines, it may be of interest to choose CAP as the primary second-line injectable drug as it will minimize the risk of AMK/KAN resistance development.<sup>203</sup> Although it is also likely that an *rrs* mutation may be acquired during a CAP-containing regimen, and thus yield resistance to both CAP and AMK/KAN, if a *tlyA* mutation is selected instead, KAN/AMK may still be considered as a choice. AMK usage as the primary second-line injectable drug is more likely to select an *rrs* mutation and derailing the use of the remaining second-line injectable drugs.<sup>203</sup>

Another alternative may lie in recovering VIO to TB treatment. VIO has been used in the past to treat TB but was later replaced by its less toxic analogue, CAP. However, and despite toxicity, VIO may prove useful in the treatment of XDR-TB as the most common mutation that confers resistance to AMK/KAN/CAP (*rrs* A1401G) does not appear to interfere with VIO mode of action.<sup>359</sup> In Lisbon, VIO might prove useful in treatment regimens directed at XDR-TB strains

belonging to the Q1 cluster. VIO is probably not active against Lisboa3 strains bearing the *tlyA* 2bp insertion as its mode of action is dependent of TlyA methyltransferase activity.<sup>365, 375</sup>

Regarding STP resistance, screening for *gidB* mutations (Chapter 6) has lead to the identification of a missense mutation associated with Q1 cluster strains. The *gidB* gene has shown to harbor lineage specific mutations.<sup>327</sup> However, to our knowledge this is the first report of an association of a *gidB* mutation with an endemic cluster associated with XDR-TB. Bioinformatic analysis shows that the mutation takes place in a conserved residue of GidB and is predicted to have an impact on protein function. Our results also support the role of this mutation in the development of an intermediate-level resistance to STP. It is also interesting to observe that no Q1 isolate has ever been detected with a *rpsL* or *rrs* mutations. Also considering that some of these isolates have tested negative for STP resistance it is possible that these have been exposed to STP due to the limited therapeutic options available. The observation that these isolates have not developed any other mutation associated with STP resistance, besides the identified *gidB* mutation, suggests that the A80P mutation in GidB seems to be enough to confer STP resistance in an *in vivo* situation.

According to the recent WHO treatment guidelines, STP is on the drug group 2.<sup>203</sup> The placement of STP in this latter group results from the fact that only one aminoglycoside/tuberactinomycin can be part of a drug regimen. WHO guidelines recommend the choice of AMK/KAN over STP due to the toxicity of the latter.<sup>203</sup> Discordant data has been published by Peloquin *et al* showing that STP was associated with lower ototoxicity when compared with AMK/KAN and, comparable nephrotoxicity with AMK, although higher than KAN.<sup>310</sup> Given the STP distinct resistance mechanism it is therefore possible to use this drug to treat drug resistant TB if STP susceptibility is documented. However, genotypic data regarding the *gidB* gene should be provided as it may have an impact on regimen efficacy. Alternatively, STP qDST may provide phenotypic data on the level of STP that will back a STP-containing regimen.

The accumulated data from genotyping of the Lisboa and Q1 strains points toward the endemicity of MDR-TB in Lisbon Health Region and uniqueness of these strains. The Lisboa and Q1 strains might represent unique examples of mycobacterial adaptation to a specific host population and anti-TB drug therapy. It became therefore necessary to characterize these strains more deeply from a phylogenetic standpoint and gain additional insights of this adaptative process at the genomic level. Moreover, a genome-wide characterization approach might enable the identification of specific strain variants that can provide clues for an

enhanced virulence or be used as specific molecular markers. Thus, for a more deep genomic study, we have, in collaboration with partners with access to NGS technologies, sequenced and analyzed the genomes of 56 *M. tuberculosis* clinical isolates, all except one recovered in Lisbon Health Region. This study, by itself, constitutes one of the largest WGS studies involving *M. tuberculosis* clinical isolates. A genome-wide SNP analysis has enabled the construction of a phylogeny showing that Lisboa3 and Q1 cluster isolates comprise two different monophyletic clades that although distinct are phylogenetically close. The main findings of this genomic study are more thoroughly discussed in Chapter 7 but it is noteworthy the identification of novel putative compensatory mutations for RIF resistance, the detection of clade specific structural variants, some of which with putative impact on strain physiology/host-pathogen interaction, or the uncovering of different substitution rates in a strain-dependent manner.

It is of special importance the identification of candidate structural variants for rapid characterization or detection of specific *M. tuberculosis* genotypes. Such genomic variants may prove especially useful in tracking descendants of original strains associated with M/XDR-TB that due to genetic diversification of traditional epidemiological markers can lead to underestimation of transmission.<sup>557</sup> In fact a 112-3 bp deletion was detected in a PPE gene that appears to be important for immune recognition.<sup>524, 525</sup> This genomic property, although not exclusive of the Lisboa3 clade may add up to other genetic traits that contribute to immune evasion and transmissibility.

The detection of structural variants is limited by the read length of the technology used in this sequencing study. Confirmation by standard PCR and sequencing will be required to confirm those structural variants that may be of interest in future studies. Therefore, structural variants specific to Lisboa3 and Q1 clades will be the subject of already ongoing studies that aim to confirm not only the structural variant itself, but also the precise genomic breakpoints. The confirmation of these variants will allow the development of a molecular assay specifically developed for tracking these strains that if implemented in the field might prove extremely useful in containing these isolates and simultaneously reduce the rates of primary M/XDR-TB in Lisbon.

The microevolution study has produced data whose analysis is still in its beginning but already allowed a glimpse of resistance acquisition dynamic and history in the two major clades. A more thorough analysis will be required to assess the putative impact of the mutations that were predicted to occur along each branch of the analysed subtrees. Lisboa3 isolates have shown a higher degree of diversification and multiple drug resistance acquisition. This

probably reflects an earlier emergence of this clade in the region. As such, upcoming studies will benefit from the inclusion of sequence data from additional Lisboa3 strains that will improve the resolution of these studies. These sort of microevolutionary studies capture the genomic adaptation process that has occurred within the population. It may be however of interest and a future objective to pursue the characterization of isolates recovered from the same patient over time. This latter approach may prove useful for the identification of genomic variants favouring a within-host adaptation process that can be performed, *e.g.*, before or after drug resistance acquisition or over a relapse episode.

Regarding the populational genetic structure, it is interesting to mention the fact that Lisboa3 and Q1 cluster isolates belong to the LAM lineage (unpublished data). More specifically, in the case of Lisboa3 cluster to the LAM1 sub-lineage whereas Q1 cluster, according to the recent rules of spoligotyping clade assignment belongs to LAM4. Analysis of the SITVITWEB database shows that 51% of the strains isolated in Portugal belong to the LAM lineage.

LAM strains are more prevalent in Africa, Central America and South America. In Europe, the more prevalent spoligotype lineages are the T and Haarlem types. Portugal therefore seems to have a significant difference in its populational structure when compared with the remainder European countries. Even comparison with Spain, the only country to which Portugal shares its borders, shows that the LAM prevalence in Spain is only 17.8%, considerably lower than in Portugal. Although these differences in the populational genetic structure support the endemism of TB in Portugal, it also poses questions regarding the phylogeographical origin of these strains.

The process of mycobacterial evolution in acquiring successive drug resistance as been highlighted in the two major clusters. It is now important, but not easy, to withdraw conclusions that ultimately lead to measures that when implemented in the field should prevent the emergence of other XDR-TB clusters. Some of the herein proposed options concerning hierarchical use of second-line injectable drugs might contribute, but also important, is to limit MDR-TB standardized treatment when no information on phenotypic resistance is available. Standardized treatment regimens applied to patients with unknown primary drug resistance can lead to resistance amplification by not providing the number of effective drugs necessary to prevent the emergence of further drug resistance.<sup>558-562</sup> The advantage of a standardized treatment consists in rapidly reducing patient infectiousness avoiding prolonged transmission. Holding the treatment might result in an increase in the number of new cases. Nevertheless, Bonilla *et al* showed that culture conversion was found to

be faster among M/XDR-TB patients with DST results dated before or less than 31 days after treatment initiation.<sup>563</sup> In this scenario molecular diagnosis of resistance might provide timely evidence to drug resistance/susceptibility which may also prove useful when no contacts with TB history are known.<sup>167, 564</sup> It is therefore important to stress the role of standard regimens as an effective way of treating TB infections by susceptible strains but, not regardless of the potential for resistance development/amplification.

In addition, the implementation of routine molecular epidemiology to new cases or at least to drug resistant cases may help to identify and contain the spreading of specific clones. As already mentioned, molecular epidemiology is highly useful when conventional contact tracing is ineffective in revealing patient links due to casual contact and identify places where transmission is taking place. Several studies show that only minor fractions of the cases showing identical genotypes were identified as contacts of the source cases.<sup>397, 565, 566</sup> The additional data provided by genotyping may guide the implementation of additional and adequate control measures.<sup>567-569</sup>

Drug resistance is predicted to carry a fitness cost that has been proposed to limit the spreading of MDR-TB by affecting its transmissibility.<sup>262, 263, 570, 571</sup> Another epidemiological model taking in account the heterogeneous fitness costs predicted that relatively fit MDR strains can eventually outcompete susceptible strains.<sup>572</sup> A more recent model incorporating molecular epidemiology data shows that although TB prevalence can decrease up to a point of local eradication, resulting from improved detection and cure rates, the proportion of drug resistant cases will increase even considering resistance fitness costs.<sup>573</sup> However, occurrence of compensatory evolution has not been taken in account in this latter model and, can have epidemiological consequences.<sup>262, 573-575</sup> Transposing these predictions to the Portuguese setting implies that active case-finding strategies especially directed at finding M/XDR-TB cases are necessary as the implemented strategies, successful in decreasing the overall TB incidence, might result in an increase of the M/XDR-TB fraction.

Despite major advances in decreasing TB incidence in Portugal, to prevent further emergence of MDR-TB clones, multidisciplinary surveillance encompassing laboratory data, traditional epidemiology and molecular epidemiology is necessary. It is important to bear in mind that there is a good reason for the fact that MDR-TB has also been described as “*Ebola with wings*”.<sup>17</sup>







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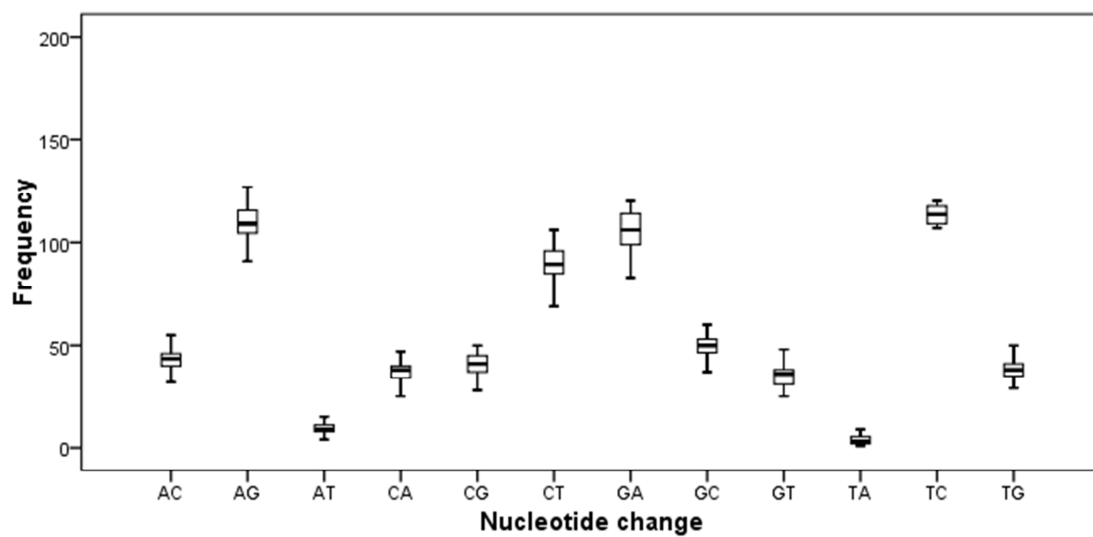
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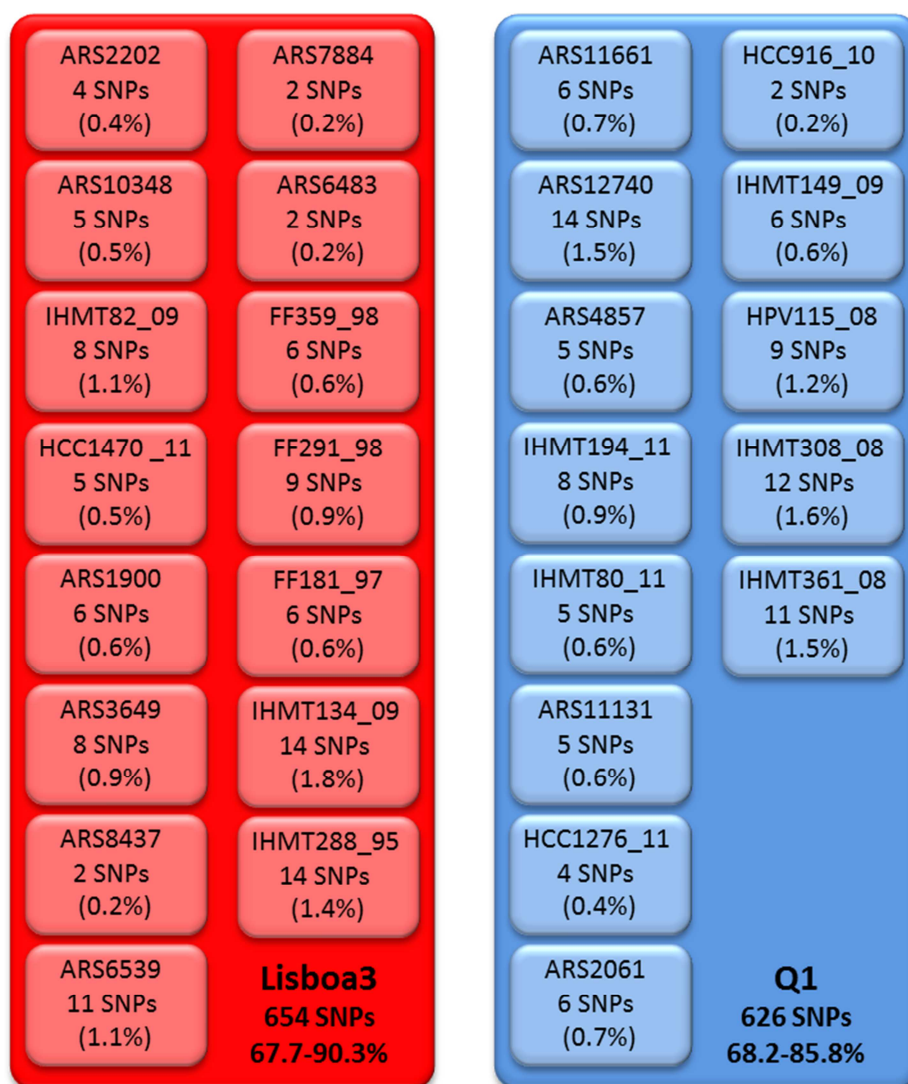


## **SUPPLEMENTARY MATERIAL**

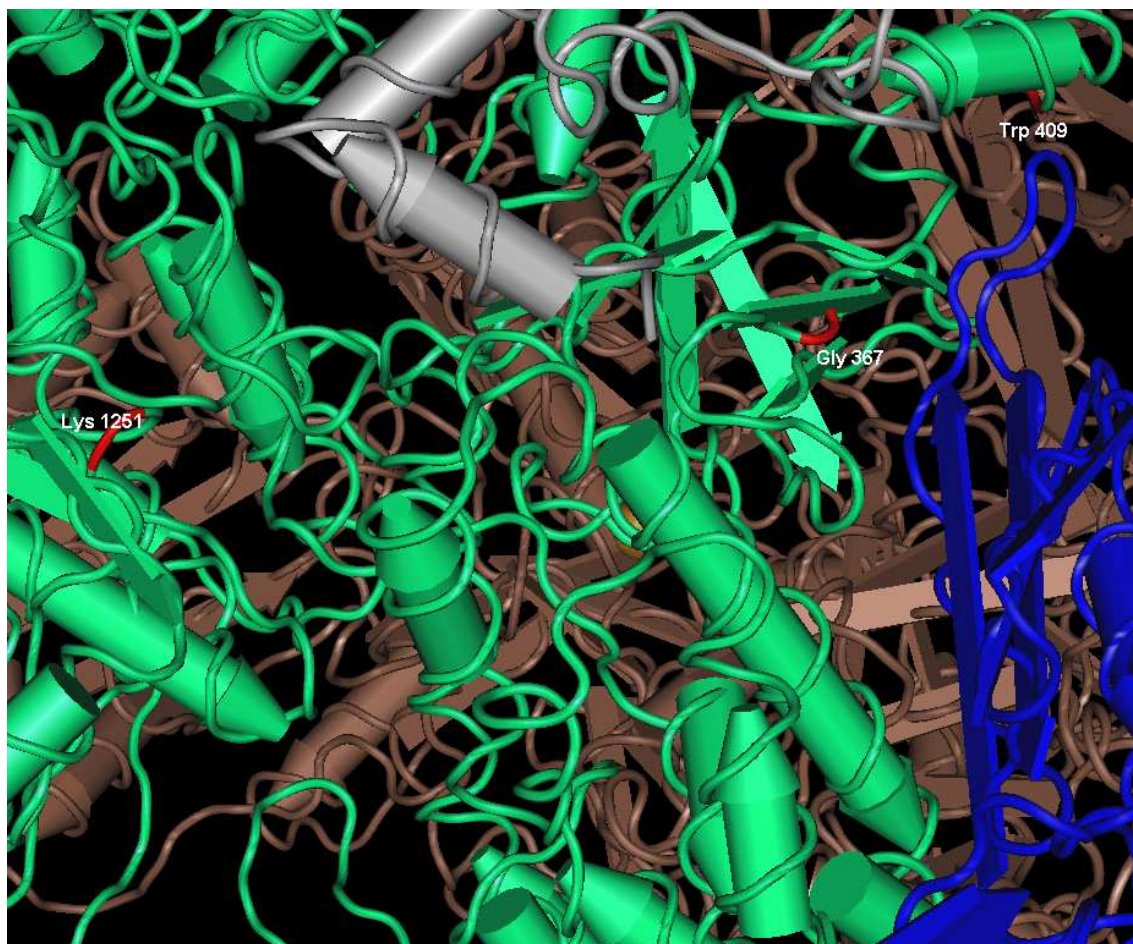




**Figure S7.1** - Boxplot graph showing the different types of SNP mutations.



**Figure S7.2** – Intra-clade SNP diversity and uniqueness. Number of SNPs unique to each isolate and percentage of total SNPs detected. Represented below each clade designation are: the number of SNPs that represents the total pool of SNPs shared by all isolates belonging to the respective clade; and, the range of the total percentage that this latter SNP pool count comprises from the total percentage of the isolates' detected SNPs.



**Figure S7.3** – Molecular model of *Escherichia coli* core RNA polymerase (Opalka et al. 2010) (RCSB Protein Data Bank ref. 3LU0) showing the homologous RpoC residues found to be involved in putative RIF resistance compensation in *M. tuberculosis*. The different RNA polymerase subunits are shown: Alpha/RpoA (blue chain), Beta/RpoB (brown chain), Beta'/RpoC (green chain) and Omega/RpoZ (grey chain). The RpoC highlighted residues, in red, Gly367, Trp409 and Lys1251 are homologous to the RpoC residues Gly442, Trp484 and Lys1152 from *M. tuberculosis*, respectively.

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[illegible]

**Figure S7.4.** Distribution of RD deletions found across the analyzed genomes of 75 *M. tuberculosis* isolates. RD absence is assigned with a black square and, Lisboa3 and Q1 clade isolates are highlighted in red and blue, respectively. Column and line totals account for the total number of RDs in column or line, respectively.

Figure S7.5

[illegible]

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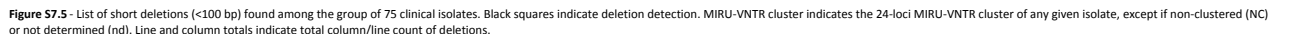


Figure S7.6

Breakpoints		Size (bp)	MRU-WTR Cluster	SCG	Isolate
Start	End				
204	205	1	rd	1	IGTB423
2724	2725	1	rd	1	ARS7886
3165	3170	1	rd	2	CC-5079
3397	3374	1	rd	2	NC2750_09
4130	4131	1	rd	2	NC2750_09
4783	4784	1	rd	2	NC2750_09
5076	5077	1	rd	2	NC2750_09
5164	5165	1	rd	2	NC2750_09
8761	8762	1	rd	2	NC2750_09
11601	11602	1	rd	2	NC2750_09
12078	12079	1	rd	2	NC2750_09
13845	13846	1	rd	2	NC2750_09
15123	15124	1	rd	2	NC2750_09
15165	15170	1	rd	2	NC2750_09
15831	15832	1	rd	2	NC2750_09
17419	17420	1	rd	2	NC2750_09
17653	17654	1	rd	2	NC2750_09
21075	21080	1	rd	2	NC2750_09
23608	23609	1	rd	2	NC2750_09
24665	24670	1	rd	2	NC2750_09
25935	25936	1	rd	2	NC2750_09
26615	26616	1	rd	2	NC2750_09
32406	32407	1	rd	2	NC2750_09
32803	32804	1	rd	2	NC2750_09
32970	32971	1	rd	2	NC2750_09
33639	33639	1	rd	2	NC2750_09
36470	36471	1	rd	2	NC2750_09
37220	37221	1	rd	2	NC2750_09
39014	39015	56	rd	2	NC2750_09
39033	39034	56	rd	2	NC2750_09
41793	41792	1	rd	2	NC2750_09
43383	43384	2	rd	2	NC2750_09
48575	48576	1	rd	2	NC2750_09
49142	49143	1	rd	2	NC2750_09
49389	49390	1	rd	2	NC2750_09
52754	52755	1	rd	2	NC2750_09
54350	54351	1	rd	2	NC2750_09
55533	55534	3	rd	2	NC2750_09
55533	55534	6	rd	2	NC2750_09
55540	55541	9	rd	2	NC2750_09
55543	55544	6	rd	2	NC2750_09
55553	55554	6	rd	2	NC2750_09
55553	55554	6	rd	2	NC2750_09
55554	55555	9	rd	2	NC2750_09
56276	56277	1	rd	2	NC2750_09
56463	56462	1	rd	2	NC2750_09
57089	57090	1	rd	2	NC2750_09
57294	57295	1	rd	2	NC2750_09
57387	57388	2	rd	2	NC2750_09
57840	57841	1	rd	2	NC2750_09
57649	57646	1	rd	2	NC2750_09
58323	58324	1	rd	2	NC2750_09
58427	58428	1	rd	2	NC2750_09
58613	58614	1	rd	2	NC2750_09
59605	59606	1	rd	2	NC2750_09
60818	60819	1	rd	2	NC2750_09
60910	60911	1	rd	2	NC2750_09
61776	61777	1	rd	2	NC2750_09
62310	62311	2	rd	2	NC2750_09
63038	63037	2	rd	2	NC2750_09
63080	63081	1	rd	2	NC2750_09
64355	64356	1	rd	2	NC2750_09
65310	65311	1	rd	2	NC2750_09
67066	67067	30	rd	2	NC2750_09
67251	67252	1	rd	2	NC2750_09
67414	67415	1	rd	2	NC2750_09
67677	67678	1	rd	2	NC2750_09
68337	68338	1	rd	2	NC2750_09
68779	68780	2	rd	2	NC2750_09
69486	69487	1	rd	2	NC2750_09
69817	69818	1	rd	2	NC2750_09
70316	70317	2	rd	2	NC2750_09
70322	70323	2	rd	2	NC2750_09
71453	71454	1	rd	2	NC2750_09
71584	71585	37	rd	2	NC2750_09
74952	74953	1	rd	2	NC2750_09
75705	75706	1	rd	2	NC2750_09
79118	79119	2	rd	2	NC2750_09
79504	79505	9	rd	2	NC2750_09
79504	79505	18	rd	2	NC2750_09
79504	79505	27	rd	2	NC2750_09
79553	79552	27	rd	2	NC2750_09
79650	79651	2	rd	2	NC2750_09
79993	80000	1	rd	2	NC2750_09
80771	80772	1	rd	2	NC2750_09
82729	82730	1	rd	2	NC2750_09
83057	83058	2	rd	2	NC2750_09
83465	83466	2	rd	2	NC2750_09
85958	85959	1	rd	2	NC2750_09
86793	86794	1	rd	2	NC2750_09
87716	87717	1	rd	2	NC2750_09
90584	90585	2	rd	2	NC2750_09
90834	90835	1	rd	2	NC2750_09
98861	98862	2	rd	2	NC2750_09
99935	99940	1	rd	2	NC2750_09
100125	100130	1	rd	2	NC2750_09
100429	100430	1	rd	2	NC2750_09
101498	101500	1	rd	2	NC2750_09
102294	102295	2	rd	2	NC2750_09
102570	102573	1	rd	2	NC2750_09
104459	104460	1	rd	2	NC2750_09
105574	105575	9	rd	2	NC2750_09
109077	109078	2	rd	2	NC2750_09
110225	110226	2	rd	2	NC2750_09
110494	110495	3	rd	2	NC2750_09
112561	112566	1	rd	2	NC2750_09
113390	113393	1	rd	2	NC2750_09
114988	114987	3	rd	2	NC2750_09
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118054	118055	1	rd	2	NC2750_09
118177	118178	1	rd	2	NC2750_09
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121946	121947	2	rd	2	NC2750_09
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152044	152045	1	rd	2	NC2750_09
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160917	160918	1	rd	2	NC2750_09
162618	162619	1	rd	2	NC2750_09
164876	164877	2	rd	2	NC2750_09
165975	165976	1	rd	2	NC2750_09
167350	167351	2	rd	2	NC2750_09
168112	168113	2	rd	2	NC2750_09
170325	170326	1	rd	2	NC2750_09
171841	171842	1	rd	2	NC2750_09
Total					18

Figure 7.6 (cont)

Breakpoints		Size (bp)		SCG	MRU-WTR Cluster	Isolate
Start	End					
174584	174585	1		rd	rd	IGB143
174780	174781	1		NC	NC	ARS7896
175942	175943	1		rd	rd	CC-5079
175925	175926	1		rd	rd	NC7250_09
179338	179339	2		rd	rd	NC7250_09
180302	180303	1		rd	rd	rd
181121	181122	2		rd	rd	rd
181342	181343	1		rd	rd	rd
182183	182184	1		rd	rd	rd
182263	182264	1		rd	rd	rd
182318	182319	1		rd	rd	rd
188288	188289	1		rd	rd	rd
190114	190115	1		rd	rd	rd
190501	190502	2		rd	rd	rd
191391	191392	1		rd	rd	rd
191885	191886	2		rd	rd	rd
193608	193609	1		rd	rd	rd
194305	194306	2		rd	rd	rd
194451	194452	1		rd	rd	rd
195183	195184	1		rd	rd	rd
195598	195599	1		rd	rd	rd
197788	197789	1		rd	rd	rd
197875	197876	1		rd	rd	rd
198424	198425	1		rd	rd	rd
198474	198475	1		rd	rd	rd
199872	199873	1		rd	rd	rd
199926	199927	1		rd	rd	rd
200208	200209	1		rd	rd	rd
201119	201120	2		rd	rd	rd
206804	206805	58		rd	rd	rd
206905	206910	58		rd	rd	rd
207820	207821	1		rd	rd	rd
210226	210227	1		rd	rd	rd
211828	211829	1		rd	rd	rd
212558	212559	1		rd	rd	rd
215718	215719	1		rd	rd	rd
218213	218214	1		rd	rd	rd
218683	218684	1		rd	rd	rd
219054	219055	1		rd	rd	rd
223628	223629	1		rd	rd	rd
223747	223748	1		rd	rd	rd
224086	224087	1		rd	rd	rd
225894	225895	3		rd	rd	rd
227270	227271	1		rd	rd	rd
228209	228210	1		rd	rd	rd
230037	230038	2		rd	rd	rd
230578	230579	1		rd	rd	rd
233190	233191	1		rd	rd	rd
234496	234497	2		rd	rd	rd
234516	234517	2		rd	rd	rd
234568	234569	1		rd	rd	rd
238511	238512	1		rd	rd	rd
239357	239358	1		rd	rd	rd
240942	240943	1		rd	rd	rd
241806	241807	1		rd	rd	rd
243144	243145	1		rd	rd	rd
246179	246180	1		rd	rd	rd
247763	247764	1		rd	rd	rd
247953	247954	1		rd	rd	rd
248213	248214	1		rd	rd	rd
248966	248967	1		rd	rd	rd
249483	249484	1		rd	rd	rd
249694	249695	2		rd	rd	rd
249746	249747	1		rd	rd	rd
253611	253612	1		rd	rd	rd
257177	257178	1		rd	rd	rd
257179	257180	2		rd	rd	rd
258605	258606	1		rd	rd	rd
259049	259050	1		rd	rd	rd
259291	259292	1		rd	rd	rd
259343	259344	1		rd	rd	rd
264093	264094	1		rd	rd	rd
264418	264419	2		rd	rd	rd
264477	264478	2		rd	rd	rd
267345	267346	2		rd	rd	rd
268164	268165	1		rd	rd	rd
268988	268989	1		rd	rd	rd
269078	269079	1		rd	rd	rd
270937	270938	1		rd	rd	rd
271348	271349	3		rd	rd	rd
271354	271355	1		rd	rd	rd
271405	271406	1		rd	rd	rd
271633	271634	1		rd	rd	rd
271727	271728	1		rd	rd	rd
271801	271802	1		rd	rd	rd
272230	272231	1		rd	rd	rd
272486	272487	1		rd	rd	rd
273138	273139	2		rd	rd	rd
273191	273192	1		rd	rd	rd
274024	274025	1		rd	rd	rd
276646	276647	1		rd	rd	rd
278110	278111	1		rd	rd	rd
278278	278279	1		rd	rd	rd
281000	281001	1		rd	rd	rd
281033	281034	2		rd	rd	rd
281115	281116	1		rd	rd	rd
281833	281834	1		rd	rd	rd
281887	281888	1		rd	rd	rd
283319	283320	1		rd	rd	rd
283758	283759	1		rd	rd	rd
284468	284469	1		rd	rd	rd
285966	285967	1		rd	rd	rd
285987	285988	1		rd	rd	rd
286808	286809	1		rd	rd	rd
288038	288039	1		rd	rd	rd
288807	288808	1		rd	rd	rd
290857	290858	2		rd	rd	rd
293364	293365	1		rd	rd	rd
293628	293629	1		rd	rd	rd
296085	296086	1		rd	rd	rd
296313	296314	1		rd	rd	rd
296543	296544	1		rd	rd	rd
297110	297111	1		rd	rd	rd
297433	297434	1		rd	rd	rd
297566	297567	2		rd	rd	rd
297972	297973	2		rd	rd	rd
300936	300937	1		rd	rd	rd
301404	301405	1		rd	rd	rd
302330	302331	1		rd	rd	rd
303166	303167	1		rd	rd	rd
304161	304162	2		rd	rd	rd
307507	307508	1		rd	rd	rd
308842	308843	1		rd	rd	rd
309174	309175	1		rd	rd	rd
309230	309231	1		rd	rd	rd
312223	312224	1		rd	rd	rd
312473	312474	1		rd	rd	rd
312914	312915	1		rd	rd	rd
314723	314724	1		rd	rd	rd
317208	317209	1		rd	rd	rd
318713	318714	1		rd	rd	rd
321664	321665	1		rd	rd	rd
324447	324448	1		rd	rd	rd
324496	324497	1		rd	rd	rd
329788	329789	1		rd	rd	rd
330366	330367	1		rd	rd	rd
330563	330564	1		rd	rd	rd
331418	331419	3		rd	rd	rd
331826	331827	2		rd	rd	rd
332870	332871	1		rd	rd	rd
333681	333682	1		rd	rd	rd
333929	333930	9		rd	rd	rd
335409	335410	10		rd	rd	rd
335682	335683	9		rd	rd	rd
335854	335855	1		rd	rd	rd
336293	336294	1		rd	rd	rd
336339	336340	1		rd	rd	rd
336652	336653	2		rd	rd	rd
336933	336934	30		rd	rd	rd
336998	336999	1		rd	rd	rd
337112	337113	1		rd	rd	rd
Total						



Figure 7.6 (cont)

Breakpoints		Size (bp)	MRU-WTR Cluster	SCG	Isolate
Start	End				
497099	497096	1	rd	1	IGTB423
497514	497515	1	NC	2	ARS7886
498943	498944	1	rd	2	CC35079
499937	499938	1	rd	2	NC2750_09
501242	501243	1	rd	2	NC2750_09
503178	503179	1	rd	2	NC2750_09
503437	503438	1	rd	2	NC2750_09
503488	503489	1	rd	2	NC2750_09
503539	503540	1	rd	2	NC2750_09
504466	504467	1	rd	2	NC2750_09
504785	504786	1	rd	2	NC2750_09
504839	504840	1	rd	2	NC2750_09
507028	507029	1	rd	2	NC2750_09
507385	507386	1	rd	2	NC2750_09
510935	510936	1	rd	2	NC2750_09
514819	514820	1	rd	2	NC2750_09
514865	514870	1	rd	2	NC2750_09
516324	516325	1	rd	2	NC2750_09
516602	516603	1	rd	2	NC2750_09
517964	517965	1	rd	2	NC2750_09
517998	517999	1	rd	2	NC2750_09
518152	518153	1	rd	2	NC2750_09
518870	518871	1	rd	2	NC2750_09
519410	519411	1	rd	2	NC2750_09
520865	520866	1	rd	2	NC2750_09
523731	523732	1	rd	2	NC2750_09
523772	523773	1	rd	2	NC2750_09
525241	525242	1	rd	2	NC2750_09
525632	525633	1	rd	2	NC2750_09
526700	526701	1	rd	2	NC2750_09
528606	528607	1	rd	2	NC2750_09
530417	530418	1	rd	2	NC2750_09
531580	531581	1	rd	2	NC2750_09
533265	533266	1	rd	2	NC2750_09
533283	533284	1	rd	2	NC2750_09
533320	533321	1	rd	2	NC2750_09
535883	535884	1	rd	2	NC2750_09
538378	538379	1	rd	2	NC2750_09
538744	538745	1	rd	2	NC2750_09
538911	538912	1	rd	2	NC2750_09
539673	539674	1	rd	2	NC2750_09
542442	542443	1	rd	2	NC2750_09
542911	542912	1	rd	2	NC2750_09
543336	543337	1	rd	2	NC2750_09
543772	543773	1	rd	2	NC2750_09
544256	544257	1	rd	2	NC2750_09
546561	546562	1	rd	2	NC2750_09
547078	547079	1	rd	2	NC2750_09
549166	549167	1	rd	2	NC2750_09
551496	551497	1	rd	2	NC2750_09
552499	552500	1	rd	2	NC2750_09
553118	553119	1	rd	2	NC2750_09
558117	558118	1	rd	2	NC2750_09
558713	558714	1	rd	2	NC2750_09
558972	558973	1	rd	2	NC2750_09
560125	560126	1	rd	2	NC2750_09
560361	560362	1	rd	2	NC2750_09
562631	562632	1	rd	2	NC2750_09
562708	562709	1	rd	2	NC2750_09
562765	562766	1	rd	2	NC2750_09
563061	563062	1	rd	2	NC2750_09
563114	563115	1	rd	2	NC2750_09
563506	563507	1	rd	2	NC2750_09
565316	565317	1	rd	2	NC2750_09
565369	565370	1	rd	2	NC2750_09
565692	565693	1	rd	2	NC2750_09
565744	565745	1	rd	2	NC2750_09
565932	565933	1	rd	2	NC2750_09
566333	566334	1	rd	2	NC2750_09
566495	566496	1	rd	2	NC2750_09
566697	566698	1	rd	2	NC2750_09
566702	566703	1	rd	2	NC2750_09
566803	566804	1	rd	2	NC2750_09
568221	568222	1	rd	2	NC2750_09
568874	568875	1	rd	2	NC2750_09
569288	569289	1	rd	2	NC2750_09
569381	569382	1	rd	2	NC2750_09
571295	571296	1	rd	2	NC2750_09
573291	573292	1	rd	2	NC2750_09
573394	573395	1	rd	2	NC2750_09
574248	574249	1	rd	2	NC2750_09
574614	574615	1	rd	2	NC2750_09
575092	575093	1	rd	2	NC2750_09
575521	575522	1	rd	2	NC2750_09
576695	576696	1	rd	2	NC2750_09
577732	577733	1	rd	2	NC2750_09
579511	579512	1	rd	2	NC2750_09
581706	581707	1	rd	2	NC2750_09
582956	582957	1	rd	2	NC2750_09
583728	583729	1	rd	2	NC2750_09
583877	583878	1	rd	2	NC2750_09
586078	586079	1	rd	2	NC2750_09
590382	590383	1	rd	2	NC2750_09
591500	591501	1	rd	2	NC2750_09
598965	598966	1	rd	2	NC2750_09
598755	598756	1	rd	2	NC2750_09
597635	597636	1	rd	2	NC2750_09
597974	597975	1	rd	2	NC2750_09
600895	600896	1	rd	2	NC2750_09
602377	602378	1	rd	2	NC2750_09
603759	603760	1	rd	2	NC2750_09
603918	603919	1	rd	2	NC2750_09
604320	604321	1	rd	2	NC2750_09
604381	604382	1	rd	2	NC2750_09
608522	608523	1	rd	2	NC2750_09
608977	608978	1	rd	2	NC2750_09
609163	609164	1	rd	2	NC2750_09
610939	610940	1	rd	2	NC2750_09
613704	613705	1	rd	2	NC2750_09
614991	614992	1	rd	2	NC2750_09
615047	615048	1	rd	2	NC2750_09
618477	618478	1	rd	2	NC2750_09
618899	618900	1	rd	2	NC2750_09
619321	619322	1	rd	2	NC2750_09
620184	620185	1	rd	2	NC2750_09
623061	623062	1	rd	2	NC2750_09
623298	623299	1	rd	2	NC2750_09
623668	623669	1	rd	2	NC2750_09
623812	623813	1	rd	2	NC2750_09
623916	623917	1	rd	2	NC2750_09
624105	624106	1	rd	2	NC2750_09
624136	624137	1	rd	2	NC2750_09
624345	624346	1	rd	2	NC2750_09
624371	624372	1	rd	2	NC2750_09
627085	627086	1	rd	2	NC2750_09
628117	628118	1	rd	2	NC2750_09
631096	631097	1	rd	2	NC2750_09
631288	631289	1	rd	2	NC2750_09
632248	632249	1	rd	2	NC2750_09
633534	633535	1	rd	2	NC2750_09
633588	633589	1	rd	2	NC2750_09
633621	633622	1	rd	2	NC2750_09
633729	633730	1	rd	2	NC2750_09
635096	635097	1	rd	2	NC2750_09
635381	635382	1	rd	2	NC2750_09
636751	636752	1	rd	2	NC2750_09
636783	636784	1	rd	2	NC2750_09
636804	636805	1	rd	2	NC2750_09
639355	639356	1	rd	2	NC2750_09
639963	639964	1	rd	2	NC2750_09
642066	642067	1	rd	2	NC2750_09
642377	642378	1	rd	2	NC2750_09
642999	642999	1	rd	2	NC2750_09
645532	645533	1	rd	2	NC2750_09
645599	645600	1	rd	2	NC2750_09
646142	646143	1	rd	2	NC2750_09
646699	646700	1	rd	2	NC2750_09
646735	646736	1	rd	2	NC2750_09
647516	647517	1	rd	2	NC2750_09
649234	649235	1	rd	2	NC2750_09
650558	650559	1	rd	2	NC2750_09
652500	652501	1	rd	2	NC2750_09
					Total

Figure 7.6 (cont)

Breakpoints		Size (bp)		SCG	Isolate
Start	End	MRU-WTR Cluster	SCG		
652999	653000	rd	1	IGTB423	
653059	653060	NC	2	ARS7886	
653128	653138	rd	2	CC35079	
653988	653989	rd	2	NC2750_09	
656842	656843	rd	2	NC2750_09	
660943	660944	rd	2	NC2750_09	
664091	664092	rd	2	NC2750_09	
664490	664491	rd	2	NC2750_09	
666609	666610	rd	2	NC2750_09	
668508	668509	rd	2	NC2750_09	
669643	669644	rd	2	NC2750_09	
670910	670911	rd	2	NC2750_09	
671024	671025	rd	2	NC2750_09	
671251	671252	rd	2	NC2750_09	
672721	672722	rd	2	NC2750_09	
673139	673140	rd	2	NC2750_09	
673308	673309	rd	2	NC2750_09	
673477	673478	rd	2	NC2750_09	
673520	673521	rd	2	NC2750_09	
673540	673541	rd	2	NC2750_09	
673781	673782	rd	2	NC2750_09	
673798	673799	rd	2	NC2750_09	
674228	674229	rd	2	NC2750_09	
674541	674542	rd	2	NC2750_09	
674945	674946	rd	2	NC2750_09	
675332	675333	rd	2	NC2750_09	
676064	676065	rd	2	NC2750_09	
676245	676246	rd	2	NC2750_09	
676559	676560	rd	2	NC2750_09	
677685	677686	rd	2	NC2750_09	
685653	685654	rd	2	NC2750_09	
686291	686292	rd	2	NC2750_09	
688213	688214	rd	2	NC2750_09	
688243	688244	rd	2	NC2750_09	
688344	688345	rd	2	NC2750_09	
688792	688793	rd	2	NC2750_09	
690336	690337	rd	2	NC2750_09	
690622	690623	rd	2	NC2750_09	
691146	691147	rd	2	NC2750_09	
691699	691700	rd	2	NC2750_09	
691883	691884	rd	2	NC2750_09	
691998	691999	rd	2	NC2750_09	
694699	694700	rd	2	NC2750_09	
698054	698055	rd	2	NC2750_09	
699128	699129	rd	2	NC2750_09	
699335	699336	rd	2	NC2750_09	
702650	702651	rd	2	NC2750_09	
702761	702762	rd	2	NC2750_09	
702846	702847	rd	2	NC2750_09	
704676	704677	rd	2	NC2750_09	
704843	704844	rd	2	NC2750_09	
706368	706369	rd	2	NC2750_09	
707936	707937	rd	2	NC2750_09	
708475	708476	rd	2	NC2750_09	
708624	708625	rd	2	NC2750_09	
708668	708669	rd	2	NC2750_09	
709601	709602	rd	2	NC2750_09	
712192	712193	rd	2	NC2750_09	
713147	713148	rd	2	NC2750_09	
715811	715812	rd	2	NC2750_09	
716000	716001	rd	2	NC2750_09	
718770	718771	rd	2	NC2750_09	
719905	719906	rd	2	NC2750_09	
719995	720000	rd	2	NC2750_09	
720392	720393	rd	2	NC2750_09	
721843	721844	rd	2	NC2750_09	
723804	723805	rd	2	NC2750_09	
723961	723962	rd	2	NC2750_09	
725098	725099	rd	2	NC2750_09	
725113	725114	rd	2	NC2750_09	
726607	726608	rd	2	NC2750_09	
727514	727515	rd	2	NC2750_09	
727872	727873	rd	2	NC2750_09	
731385	731386	rd	2	NC2750_09	
731593	731594	rd	2	NC2750_09	
732650	732651	rd	2	NC2750_09	
732868	732869	rd	2	NC2750_09	
733067	733068	rd	2	NC2750_09	
735785	735786	rd	2	NC2750_09	
735912	735913	rd	2	NC2750_09	
737223	737224	rd	2	NC2750_09	
739626	739627	rd	2	NC2750_09	
740182	740183	rd	2	NC2750_09	
741675	741676	rd	2	NC2750_09	
745285	745286	rd	2	NC2750_09	
746327	746328	rd	2	NC2750_09	
746333	746334	rd	2	NC2750_09	
748521	748522	rd	2	NC2750_09	
749225	749226	rd	2	NC2750_09	
750440	750441	rd	2	NC2750_09	
750492	750493	rd	2	NC2750_09	
751455	751456	rd	2	NC2750_09	
753809	753810	rd	2	NC2750_09	
753899	753900	rd	2	NC2750_09	
753995	753996	rd	2	NC2750_09	
754410	754411	rd	2	NC2750_09	
760061	760062	rd	2	NC2750_09	
760854	760855	rd	2	NC2750_09	
761341	761342	rd	2	NC2750_09	
763163	763164	rd	2	NC2750_09	
765210	765211	rd	2	NC2750_09	
765668	765669	rd	2	NC2750_09	
767145	767146	rd	2	NC2750_09	
768878	768879	rd	2	NC2750_09	
769028	769029	rd	2	NC2750_09	
773361	773362	rd	2	NC2750_09	
773672	773673	rd	2	NC2750_09	
773932	773933	rd	2	NC2750_09	
775322	775323	rd	2	NC2750_09	
775908	775909	rd	2	NC2750_09	
779183	779184	rd	2	NC2750_09	
781663	781664	rd	2	NC2750_09	
781715	781716	rd	2	NC2750_09	
783936	783937	rd	2	NC2750_09	
784273	784274	rd	2	NC2750_09	
787543	787544	rd	2	NC2750_09	
790665	790666	rd	2	NC2750_09	
791060	791061	rd	2	NC2750_09	
792303	792304	rd	2	NC2750_09	
792495	792496	rd	2	NC2750_09	
792703	792704	rd	2	NC2750_09	
792759	792760	rd	2	NC2750_09	
793972	793973	rd	2	NC2750_09	
801305	801306	rd	2	NC2750_09	
802448	802449	rd	2	NC2750_09	
804260	804261	rd	2	NC2750_09	
804312	804313	rd	2	NC2750_09	
805576	805577	rd	2	NC2750_09	
806707	806708	rd	2	NC2750_09	
809123	809124	rd	2	NC2750_09	
809846	809847	rd	2	NC2750_09	
809932	809933	rd	2	NC2750_09	
810583	810584	rd	2	NC2750_09	
812866	812867	rd	2	NC2750_09	
813242	813243	rd	2	NC2750_09	
813281	813282	rd	2	NC2750_09	
813874	813875	rd	2	NC2750_09	
815307	815308	rd	2	NC2750_09	
821590	821591	rd	2	NC2750_09	
825746	825747	rd	2	NC2750_09	
825771	825772	rd	2	NC2750_09	
825774	825775	rd	2	NC2750_09	
826808	826809	rd	2	NC2750_09	
828150	828151	rd	2	NC2750_09	
828198	828199	rd	2	NC2750_09	
829053	829054	rd	2	NC2750_09	
829274	829275	rd	2	NC2750_09	
830307	830308	rd	2	NC2750_09	
830866	830867	rd	2	NC2750_09	
830890	830891	rd	2	NC2750_09	
831896	831897	rd	2	NC2750_09	
832699	832700	rd	2	NC2750_09	
Total					1



Figure 7.6 (cont)

Breakpoints		Size (bp)		SCG	Isolate																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																	
Start	End	MRSA-VR Cluster	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144	145	146	147	148	149	150	151	152	153	154	155	156	157	158	159	160	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175	176	177	178	179	180	181	182	183	184	185	186	187	188	189	190	191	192	193	194	195	196	197	198	199	200	201	202	203	204	205	206	207	208	209	210	211	212	213	214	215	216	217	218	219	220	221	222	223	224	225	226	227	228	229	230	231	232	233	234	235	236	237	238	239	240	241	242	243	244	245	246	247	248	249	250	251	252	253	254	255	256	257	258	259	260	261	262	263	264	265	266	267	268	269	270	271	272	273	274	275	276	277	278	279	280	281	282	283	284	285	286	287	288	289	290	291	292	293	294	295	296	297	298	299	300	301	302	303	304	305	306	307	308	309	310	311	312	313	314	315	316	317	318	319	320	321	322	323	324	325	326	327	328	329	330	331	332	333	334	335	336	337	338	339	340	341	342	343	344	345	346	347	348	349	350	351	352	353	354	355	356	357	358	359	360	361	362	363	364	365	366	367	368	369	370	371	372	373	374	375	376	377	378	379	380	381	382	383	384	385	386	387	388	389	390	391	392	393	394	395	396	397	398	399	400	401	402	403	404	405	406	407	408	409	410	411	412	413	414	415	416	417	418	419	420	421	422	423	424	425	426	427	428	429	430	431	432	433	434	435	436	437	438	439	440	441	442	443	444	445	446	447	448	449	450	451	452	453	454	455	456	457	458	459	460	461	462	463	464	465	466	467	468	469	470	471	472	473	474	475	476	477	478	479	480	481	482	483	484	485	486	487	488	489	490	491	492	493	494	495	496	497	498	499	500	501	502	503	504	505	506	507	508	509	510	511	512	513	514	515	516	517	518	519	520	521	522	523	524	525	526	527	528	529	530	531	532	533	534	535	536	537	538	539	540	541	542	543	544	545	546	547	548	549	550	551	552	553	554	555	556	557	558	559	560	561	562	563	564	565	566	567	568	569	570	571	572	573	574	575	576	577	578	579	580	581	582	583	584	585	586	587	588	589	590	591	592	593	594	595	596	597	598	599	600	601	602	603	604	605	606	607	608	609	610	611	612	613	614	615	616	617	618	619	620	621	622	623	624	625	626	627	628	629	630	631	632	633	634	635	636	637	638	639	640	641	642	643	644	645	646	647	648	649	650	651	652	653	654	655	656	657	658	659	660	661	662	663	664	665	666	667	668	669	670	671	672	673	674	675	676	677	678	679	680	681	682	683	684	685	686	687	688	689	690	691	692	693	694	695	696	697	698	699	700	701	702	703	704	705	706	707	708	709	710	711	712	713	714	715	716	717	718	719	720	721	722	723	724	725	726	727	728	729	730	731	732	733	734	735	736	737	738	739	740	741	742	743	744	745	746	747	748	749	750	751	752	753	754	755	756	757	758	759	760	761	762	763	764	765	766	767	768	769	770	771	772	773	774	775	776	777	778	779	780	781	782	783	784	785	786	787	788	789	790	791	792	793	794	795	796	797	798	799	800	801	802	803	804	805	806	807	808	809	810	811	812	813	814	815	816	817	818	819	820	821	822	823	824	825	826	827	828	829	830	831	832	833	834	835	836	837	838	839	840	841	842	843	844	845	846	847	848	849	850	851	852	853	854	855	856	857	858	859	860	861	862	863	864	865	866	867	868	869	870	871	872	873	874	875	876	877	878	879	880	881	882	883	884	885	886	887	888	889	890	891	892	893	894	895	896	897	898	899	900	901	902	903	904	905	906	907	908	909	910	911	912	913	914	915	916	917	918	919	920	921	922	923	924	925	926	927	928	929	930	931	932	933	934	935	936	937	938	939	940	941	942	943	944	945	946	947	948	949	950	951	952	953	954	955	956	957	958	959	960	961	962	963	964	965	966	967	968	969	970	971	972	973	974	975	976	977	978	979	980	981	982	983	984	985	986	987	988	989	990	991	992	993	994	995	996	997	998	999	1000	1001	1002	1003	1004	1005	1006	1007	1008	1009	1010	1011	1012	1013	1014	1015	1016	1017	1018	1019	1020	1021	1022	1023	1024	1025	1026	1027	1028	1029	1030	1031	1032	1033	1034	1035	1036	1037	1038	1039	1040	1041	1042	1043	1044	1045	1046	1047	1048	1049	1050	1051	1052	1053	1054	1055	1056	1057	1058	1059	1060	1061	1062	1063	1064	1065	1066	1067	1068	1069	1070	1071	1072	1073	1074	1075	1076	1077	1078	1079	1080	1081	1082	1083	1084	1085	1086	1087	1088	1089	1090	1091	1092	1093	1094	1095	1096	1097	1098	1099	1100	1101	1102	1103	1104	1105	1106	1107	1108	1109	1110	1111	1112	1113	1114	1115	1116	1117	1118	1119	1120	1121	1122	1123	1124	1125	1126	1127	1128	1129	1130	1131	1132	1133	1134	1135	1136	1137	1138	1139	1140	1141	1142	1143	1144	1145	1146	1147	1148	1149	1150	1151	1152	1153	1154	1155	1156	1157	1158	1159	1160	1161	1162	1163	1164	1165	1166	1167	1168	1169	1170	1171	1172	1173	1174	1175	1176	1177	1178	1179	1180	1181	1182	1183	1184	1185	1186	1187	1188	1189	1190	1191	1192	1193	1194	1195	1196	1197	1198	1199	1200	1201	1202	1203	1204	1205	1206	1207	1208	1209	1210	1211	1212	1213	1214	1215	1216	1217	1218	1219	1220	1221	1222	1223	1224	1225	1226	1227	1228	1229	1230	1231	1232	1233	1234	1235	1236	1237	1238	1239	1240	1241	1242	1243	1244	1245	1246	1247	1248	1249	1250	1251	1252	1253	1254	1255	1256	1257	1258	1259	1260	1261	1262	1263	1264	1265	1266	1267	1268	1269	1270	1271	1272	1273	1274	1275	1276	1277	1278	1279	1280	1281	1282	1283	1284	1285	1286	1287	1288	1289	1290	1291	1292	1293	1294	1295	1296	1297	1298	1299	1300	1301	1302	1303	1304	1305	1306	1307	1308	1309	1310	1311	1312	1313	1314	1315	1316	1317	1318	1319	1320	1321	1322	1323	1324	1325	1326	1327	1328	1329	1330	1331	1332	1333	1334	1335	1336	1337	1338	1339	1340	1341	1342	1343	1344	1345	1346	1347	1348	1349	1350	1351	1352	1353	1354	1355	1356	1357	1358	1359	1360	1361	1362	1363	1364	1365	1366	1367	1368	1369	1370	1371	1372	1373	1374	1375	1376	1377	1378	1379	1380	1381	1382	1383	1384	1385	1386	1387	1388	1389	1390	1391	1392	1393	1394	1395	1396	1397	1398	1399	1400	1401	1402	1403	1404	1405	1406	1407	1408	1409	1410	1411	1412	1413	1414	1415	1416	1417	1418	1419	1420	1421	1422	1423	1424	1425	1426	1427	1428	1429	1430	1431	1432	1433	1434	1435	1436	1437	1438	1439	1440	1441	1442	1443	1444	1445	1446	1447	1448	1449	1450	1451	1452	1453	1454	1455	1456	1457	1458	1459	1460	1461	1462	1463	1464	1465	1466	1467	1468	1469	1470	1471	1472	1473	1474	1475	1476	1477	1478	1479	1480	1481	1482	1483	1484	1485	1486	1487	1488	1489	1490	1491	149



Figure 7.6 (cont)

Breakpoints		Size (bp)		SCG	Isolate
Start	End	MIRU-VNR Cluster			
1090188	1090188	1	rd	1	IGB423
1090972	1090972	2	NC	2	ARS7896
1091093	1091093	3	NC	3	CC35079
1091498	1091498	4	rd	4	NC250_09
1091562	1091562	10	NC	10	NC250_09
1091605	1091605	1	rd	1	NC250_09
1091733	1091733	10	rd	10	NC250_09
1091888	1091888	1	rd	1	NC250_09
1091982	1091982	12	rd	12	NC250_09
1092346	1092346	1	rd	1	NC250_09
1092445	1092445	1	rd	1	NC250_09
1093064	1093064	2	rd	2	NC250_09
1093444	1093444	1	rd	1	NC250_09
1093685	1093685	1	rd	1	NC250_09
1093907	1093907	31	rd	31	NC250_09
1093998	1093998	2	rd	2	NC250_09
1094094	1094094	1	rd	1	NC250_09
1094756	1094756	25	rd	25	NC250_09
1095888	1095888	3	rd	3	NC250_09
1096263	1096263	20	rd	20	NC250_09
1097821	1097821	1	rd	1	NC250_09
1098999	1098999	1	rd	1	NC250_09
1100908	1100908	1	rd	1	NC250_09
1101094	1101094	1	rd	1	NC250_09
1101763	1101763	1	rd	1	NC250_09
1103486	1103486	3	rd	3	NC250_09
1103555	1103555	1	rd	1	NC250_09
1105042	1105042	1	rd	1	NC250_09
1105543	1105543	1	rd	1	NC250_09
1105997	1105997	4	rd	4	NC250_09
1110271	1110271	1	rd	1	NC250_09
1110426	1110426	1	rd	1	NC250_09
1110862	1110862	1	rd	1	NC250_09
1112922	1112922	6	rd	6	NC250_09
1113610	1113610	6	rd	6	NC250_09
1113614	1113614	6	rd	6	NC250_09
1114160	1114160	1	rd	1	NC250_09
1114258	1114258	1	rd	1	NC250_09
1117344	1117344	1	rd	1	NC250_09
1119753	1119753	1	rd	1	NC250_09
1119813	1119813	1	rd	1	NC250_09
1120788	1120788	1	rd	1	NC250_09
1121653	1121653	15	rd	15	NC250_09
1121806	1121806	1	rd	1	NC250_09
1123333	1123333	1	rd	1	NC250_09
1123352	1123352	1	rd	1	NC250_09
1129352	1129352	1	rd	1	NC250_09
1130375	1130375	1	rd	1	NC250_09
1131226	1131226	1	rd	1	NC250_09
1132963	1132963	1	rd	1	NC250_09
1136526	1136526	1	rd	1	NC250_09
1145555	1145555	1	rd	1	NC250_09
1146846	1146846	1	rd	1	NC250_09
1151849	1151849	2	rd	2	NC250_09
1152968	1152968	1	rd	1	NC250_09
1155144	1155144	1	rd	1	NC250_09
1155464	1155464	1	rd	1	NC250_09
1156136	1156136	1	rd	1	NC250_09
1157032	1157032	1	rd	1	NC250_09
1160213	1160213	1	rd	1	NC250_09
1160688	1160688	1	rd	1	NC250_09
1161444	1161444	1	rd	1	NC250_09
1162899	1162899	2	rd	2	NC250_09
1165523	1165523	1	rd	1	NC250_09
1165774	1165774	1	rd	1	NC250_09
1166218	1166218	2	rd	2	NC250_09
1166562	1166562	1	rd	1	NC250_09
1168178	1168178	2	rd	2	NC250_09
1168715	1168715	1	rd	1	NC250_09
1169308	1169308	1	rd	1	NC250_09
1171072	1171072	1	rd	1	NC250_09
1171998	1171998	1	rd	1	NC250_09
1172678	1172678	3	rd	3	NC250_09
1173092	1173092	2	rd	2	NC250_09
1177975	1177975	4	rd	4	NC250_09
1179186	1179186	2	rd	2	NC250_09
1180593	1180593	1	rd	1	NC250_09
1184403	1184403	1	rd	1	NC250_09
1187524	1187524	1	rd	1	NC250_09
1187724	1187724	1	rd	1	NC250_09
1188773	1188773	10	rd	10	NC250_09
1188793	1188793	24	rd	24	NC250_09
1188925	1188925	1	rd	1	NC250_09
1189005	1189005	10	rd	10	NC250_09
1189054	1189054	1	rd	1	NC250_09
1189063	1189063	1	rd	1	NC250_09
1189099	1189099	1	rd	1	NC250_09
1189133	1189133	18	rd	18	NC250_09
1189286	1189286	5	rd	5	NC250_09
1189325	1189325	1	rd	1	NC250_09
1189814	1189814	1	rd	1	NC250_09
1190208	1190208	1	rd	1	NC250_09
1190874	1190874	1	rd	1	NC250_09
1192053	1192053	1	rd	1	NC250_09
1196663	1196663	1	rd	1	NC250_09
1201114	1201114	1	rd	1	NC250_09
1201207	1201207	1	rd	1	NC250_09
1203366	1203366	1	rd	1	NC250_09
1203504	1203504	3	rd	3	NC250_09
1204963	1204963	1	rd	1	NC250_09
1204998	1204998	1	rd	1	NC250_09
1206535	1206535	1	rd	1	NC250_09
1206612	1206612	1	rd	1	NC250_09
1207743	1207743	2	rd	2	NC250_09
1207894	1207894	1	rd	1	NC250_09
1207945	1207945	1	rd	1	NC250_09
1210345	1210345	1	rd	1	NC250_09
1211844	1211844	1	rd	1	NC250_09
1212303	1212303	1	rd	1	NC250_09
1212322	1212322	1	rd	1	NC250_09
1212410	1212410	1	rd	1	NC250_09
1212422	1212422	1	rd	1	NC250_09
1212636	1212636	18	rd	18	NC250_09
1212811	1212811	3	rd	3	NC250_09
1212876	1212876	1	rd	1	NC250_09
1213245	1213245	2	rd	2	NC250_09
1213386	1213386	1	rd	1	NC250_09
1213495	1213495	7	rd	7	NC250_09
1213602	1213602	5	rd	5	NC250_09
1213671	1213671	45	rd	45	NC250_09
1213886	1213886	1	rd	1	NC250_09
1214783	1214783	2	rd	2	NC250_09
1215753	1215753	1	rd	1	NC250_09
1217171	1217171	1	rd	1	NC250_09
1217642	1217642	3	rd	3	NC250_09
1217993	1217993	18	rd	18	NC250_09
1217998	1217998	6	rd	6	NC250_09
1218933	1218933	1	rd	1	NC250_09
1220333	1220333	1	rd	1	NC250_09
1225482	1225482	2	rd	2	NC250_09
1226353	1226353	1	rd	1	NC250_09
1226403	1226403	2	rd	2	NC250_09
1227630	1227630	1	rd	1	NC250_09
1227893	1227893	1	rd	1	NC250_09
1227942	1227942	1	rd	1	NC250_09
1228873	1228873	3	rd	3	NC250_09
1228918	1228918	1	rd	1	NC250_09
1228993	1228993	1	rd	1	NC250_09
1230501	1230501	1	rd	1	NC250_09
1230999	1230999	1	rd	1	NC250_09
1231265	1231265	1	rd	1	NC250_09
1232586	1232586	1	rd	1	NC250_09
1233913	1233913	1	rd	1	NC250_09
1236833	1236833	1	rd	1	NC250_09
1237853	1237853	1	rd	1	NC250_09
1237913	1237913	1	rd	1	NC250_09
1238446	1238446	1	rd	1	NC250_09
1239728	1239728	1	rd	1	NC250_09
1240241	1240241	1	rd	1	NC250_09
1240293	1240293	1	rd	1	NC250_09
1241772	1241772	1	rd	1	NC250_09
1242194	1242194	1	rd	1	NC250_09
Total					1

Figure 7.6 (cont)

Breakpoints		Size (bp)	SCG	Isolate
Start	End		MRU-WTR Cluster	
1244753	1244754	1	rd	IGTB423
1249053	1249052	1	NC	ARS7896
1251633	1251632	1	rd	CC35079
1252433	1252432	1	NC	NC2562_09
1255193	1255192	1	rd	NC2562_09
1255443	1255442	1	rd	NC2562_09
1258958	1258957	39	rd	NC2562_09
1260633	1260632	2	rd	NC2562_09
1260688	1260687	1	rd	NC2562_09
1261558	1261557	1	rd	NC2562_09
1263004	1263003	1	rd	NC2562_09
1263885	1263884	1	rd	NC2562_09
1264055	1264054	1	rd	NC2562_09
1264363	1264362	3	rd	NC2562_09
1264783	1264782	1	rd	NC2562_09
1265210	1265209	1	rd	NC2562_09
1265422	1265421	1	rd	NC2562_09
1266583	1266582	1	rd	NC2562_09
1267143	1267142	1	rd	NC2562_09
1267893	1267892	1	rd	NC2562_09
1269799	1269798	1	rd	NC2562_09
1270206	1270205	1	rd	NC2562_09
1270366	1270365	1	rd	NC2562_09
1271143	1271142	1	rd	NC2562_09
1272115	1272114	2	rd	NC2562_09
1272652	1272651	1	rd	NC2562_09
1272878	1272877	1	rd	NC2562_09
1273250	1273249	1	rd	NC2562_09
1274453	1274452	2	rd	NC2562_09
1276803	1276802	2	rd	NC2562_09
1276886	1276885	1	rd	NC2562_09
1276903	1276902	1	rd	NC2562_09
1277443	1277442	1	rd	NC2562_09
1277868	1277867	1	rd	NC2562_09
1278833	1278832	1	rd	NC2562_09
1278914	1278913	1	rd	NC2562_09
1279145	1279144	1	rd	NC2562_09
1279693	1279692	1	rd	NC2562_09
1280683	1280682	6	rd	NC2562_09
1281993	1281992	1	rd	NC2562_09
1284423	1284422	1	rd	NC2562_09
1285033	1285032	1	rd	NC2562_09
1285888	1285887	1	rd	NC2562_09
1285943	1285942	1	rd	NC2562_09
1287428	1287427	1	rd	NC2562_09
1289165	1289164	2	rd	NC2562_09
1289493	1289492	1	rd	NC2562_09
1291148	1291147	1	rd	NC2562_09
1291211	1291210	1	rd	NC2562_09
1295706	1295705	2	rd	NC2562_09
1295812	1295811	1	rd	NC2562_09
1296811	1296810	1	rd	NC2562_09
1296863	1296862	1	rd	NC2562_09
1296914	1296913	1	rd	NC2562_09
1303196	1303195	1	rd	NC2562_09
1306086	1306085	2	rd	NC2562_09
1306623	1306622	1	rd	NC2562_09
1307998	1307997	1	rd	NC2562_09
1308218	1308217	1	rd	NC2562_09
1309945	1309944	1	rd	NC2562_09
1309996	1309995	1	rd	NC2562_09
1310275	1310274	1	rd	NC2562_09
1312627	1312626	1	rd	NC2562_09
1313363	1313362	1	rd	NC2562_09
1316313	1316312	1	rd	NC2562_09
1317981	1317980	1	rd	NC2562_09
1317898	1317897	1	rd	NC2562_09
1318136	1318135	1	rd	NC2562_09
1320915	1320914	1	rd	NC2562_09
1322143	1322142	1	rd	NC2562_09
1323385	1323384	1	rd	NC2562_09
1323881	1323880	1	rd	NC2562_09
1324395	1324394	1	rd	NC2562_09
1329074	1329073	1	rd	NC2562_09
1329373	1329372	1	rd	NC2562_09
1329554	1329553	1	rd	NC2562_09
1332418	1332417	1	rd	NC2562_09
1332536	1332535	1	rd	NC2562_09
1333663	1333662	1	rd	NC2562_09
1333863	1333862	1	rd	NC2562_09
1334283	1334282	1	rd	NC2562_09
1334683	1334682	1	rd	NC2562_09
1334724	1334723	1	rd	NC2562_09
1334998	1334997	2	rd	NC2562_09
1335173	1335172	1	rd	NC2562_09
1335493	1335492	1	rd	NC2562_09
1335505	1335504	1	rd	NC2562_09
1337324	1337323	1	rd	NC2562_09
1338999	1338998	2	rd	NC2562_09
1339811	1339810	1	rd	NC2562_09
1340230	1340229	12	rd	NC2562_09
1340993	1340992	1	rd	NC2562_09
1342715	1342714	1	rd	NC2562_09
1343078	1343077	1	rd	NC2562_09
1343140	1343139	1	rd	NC2562_09
1343195	1343194	1	rd	NC2562_09
1343318	1343317	1	rd	NC2562_09
1344298	1344297	1	rd	NC2562_09
1345953	1345952	1	rd	NC2562_09
1350473	1350472	1	rd	NC2562_09
1351818	1351817	1	rd	NC2562_09
1353178	1353177	2	rd	NC2562_09
1353718	1353717	1	rd	NC2562_09
1354388	1354387	1	rd	NC2562_09
1355866	1355865	1	rd	NC2562_09
1357249	1357248	1	rd	NC2562_09
1357468	1357467	1	rd	NC2562_09
1357781	1357780	1	rd	NC2562_09
1358548	1358547	3	rd	NC2562_09
1358943	1358942	2	rd	NC2562_09
1360124	1360123	2	rd	NC2562_09
1360375	1360374	1	rd	NC2562_09
1360960	1360959	1	rd	NC2562_09
1365211	1365210	2	rd	NC2562_09
1365288	1365287	3	rd	NC2562_09
1365813	1365812	1	rd	NC2562_09
1365833	1365832	2	rd	NC2562_09
1366308	1366307	1	rd	NC2562_09
1367818	1367817	1	rd	NC2562_09
1368322	1368321	1	rd	NC2562_09
1369613	1369612	1	rd	NC2562_09
1370585	1370584	1	rd	NC2562_09
1371963	1371962	2	rd	NC2562_09
1373112	1373111	2	rd	NC2562_09
1375459	1375458	1	rd	NC2562_09
1375538	1375537	1	rd	NC2562_09
1376663	1376662	1	rd	NC2562_09
1381938	1381937	1	rd	NC2562_09
1384493	1384492	3	rd	NC2562_09
1385268	1385267	1	rd	NC2562_09
1385408	1385407	1	rd	NC2562_09
1385524	1385523	1	rd	NC2562_09
1386158	1386157	1	rd	NC2562_09
1386260	1386259	4	rd	NC2562_09
1386293	1386292	1	rd	NC2562_09
1387416	1387415	1	rd	NC2562_09
1392153	1392152	1	rd	NC2562_09
1392893	1392892	1	rd	NC2562_09
1392999	1392998	1	rd	NC2562_09
1393715	1393714	1	rd	NC2562_09
1394475	1394474	1	rd	NC2562_09
1394533	1394532	1	rd	NC2562_09
1395182	1395181	3	rd	NC2562_09
1395453	1395452	2	rd	NC2562_09
1396186	1396185	1	rd	NC2562_09
1396538	1396537	1	rd	NC2562_09
1397288	1397287	2	rd	NC2562_09
1398804	1398803	2	rd	NC2562_09
1399486	1399485	1	rd	NC2562_09
1401750	1401749	1	rd	NC2562_09
Total			18	NC

Breakpoints		Size (bp)	MRU-WTR		SCG	Isolate
Start	End		Cluster			
1405087	1405088	1	rd	1	rd	IGTB423
1406062	1406068	1	NC	2	NC	ARS7886
1406760	1406765	1	rd	2	rd	CC-53078
1413374	1413375	2	rd	2	rd	NC2752_09
1413872	1413875	1	rd	2	rd	NC2752_09
1418393	1418394	1	rd	2	rd	NC2752_09
1418446	1418447	1	rd	2	rd	NC2752_09
1418863	1418864	8	rd	2	rd	NC2752_09
1419035	1419036	1	rd	2	rd	NC2752_09
1422064	1422065	1	rd	2	rd	NC2752_09
1423070	1423071	1	rd	2	rd	NC2752_09
1423836	1423837	1	rd	2	rd	NC2752_09
1426886	1426887	1	rd	2	rd	NC2752_09
1429171	1429181	1	rd	2	rd	NC2752_09
1430638	1430639	1	rd	2	rd	NC2752_09
1431320	1431321	1	rd	2	rd	NC2752_09
1431495	1431496	1	rd	2	rd	NC2752_09
1433900	1433901	1	rd	2	rd	NC2752_09
1433950	1433951	1	rd	2	rd	NC2752_09
1438241	1438242	1	rd	2	rd	NC2752_09
1440091	1440094	1	rd	2	rd	NC2752_09
1441553	1441555	1	rd	2	rd	NC2752_09
1442577	1442578	1	rd	2	rd	NC2752_09
1442640	1442641	1	rd	2	rd	NC2752_09
1443428	1443429	56	rd	2	rd	NC2752_09
1443483	1443484	1	rd	2	rd	NC2752_09
1448333	1448334	1	rd	2	rd	NC2752_09
1448514	1448515	1	rd	2	rd	NC2752_09
1449450	1449451	1	rd	2	rd	NC2752_09
1450843	1450844	1	rd	2	rd	NC2752_09
1450964	1450965	1	rd	2	rd	NC2752_09
1451033	1451034	1	rd	2	rd	NC2752_09
1453226	1453227	1	rd	2	rd	NC2752_09
1455732	1455733	1	rd	2	rd	NC2752_09
1455894	1455895	1	rd	2	rd	NC2752_09
1457308	1457309	1	rd	2	rd	NC2752_09
1458042	1458043	1	rd	2	rd	NC2752_09
1460981	1460982	1	rd	2	rd	NC2752_09
1462462	1462463	1	rd	2	rd	NC2752_09
1463578	1463581	1	rd	2	rd	NC2752_09
1463584	1463585	2	rd	2	rd	NC2752_09
1466523	1466524	1	rd	2	rd	NC2752_09
1466564	1466565	2	rd	2	rd	NC2752_09
1466978	1466979	1	rd	2	rd	NC2752_09
1471176	1471177	1	rd	2	rd	NC2752_09
1471178	1471179	3	rd	2	rd	NC2752_09
1471285	1471290	1	rd	2	rd	NC2752_09
1471335	1471340	2	rd	2	rd	NC2752_09
1472653	1472658	1	rd	2	rd	NC2752_09
1472910	1472911	1	rd	2	rd	NC2752_09
1472920	1472921	1	rd	2	rd	NC2752_09
1473384	1473385	1	rd	2	rd	NC2752_09
1473551	1473552	1	rd	2	rd	NC2752_09
1474161	1474162	1	rd	2	rd	NC2752_09
1474609	1474610	1	rd	2	rd	NC2752_09
1474626	1474627	1	rd	2	rd	NC2752_09
1475876	1475877	1	rd	2	rd	NC2752_09
1475953	1475958	1	rd	2	rd	NC2752_09
1477186	1477187	1	rd	2	rd	NC2752_09
1478211	1478212	2	rd	2	rd	NC2752_09
1479428	1479429	1	rd	2	rd	NC2752_09
1479584	1479585	1	rd	2	rd	NC2752_09
1480505	1480506	1	rd	2	rd	NC2752_09
1485508	1485509	1	rd	2	rd	NC2752_09
1485673	1485674	1	rd	2	rd	NC2752_09
1485999	1485999	1	rd	2	rd	NC2752_09
1487654	1487655	2	rd	2	rd	NC2752_09
1487808	1487809	1	rd	2	rd	NC2752_09
1487953	1487954	2	rd	2	rd	NC2752_09
1488585	1488586	1	rd	2	rd	NC2752_09
1488926	1488927	11	rd	2	rd	NC2752_09
1489302	1489303	1	rd	2	rd	NC2752_09
1489608	1489609	1	rd	2	rd	NC2752_09
1489724	1489725	1	rd	2	rd	NC2752_09
1489915	1489916	1	rd	2	rd	NC2752_09
1492044	1492045	1	rd	2	rd	NC2752_09
1495533	1495534	1	rd	2	rd	NC2752_09
1502466	1502467	1	rd	2	rd	NC2752_09
1503953	1503954	2	rd	2	rd	NC2752_09
1504265	1504266	1	rd	2	rd	NC2752_09
1504956	1504957	1	rd	2	rd	NC2752_09
1505604	1505605	1	rd	2	rd	NC2752_09
1510120	1510121	1	rd	2	rd	NC2752_09
1511914	1511915	1	rd	2	rd	NC2752_09
1516205	1516206	1	rd	2	rd	NC2752_09
1517106	1517107	1	rd	2	rd	NC2752_09
1517166	1517167	1	rd	2	rd	NC2752_09
1517972	1517973	1	rd	2	rd	NC2752_09
1517975	1517976	1	rd	2	rd	NC2752_09
1518746	1518747	1	rd	2	rd	NC2752_09
1519536	1519537	1	rd	2	rd	NC2752_09
1521595	1521596	1	rd	2	rd	NC2752_09
1523078	1523079	1	rd	2	rd	NC2752_09
1524307	1524308	1	rd	2	rd	NC2752_09
1526999	1526999	1	rd	2	rd	NC2752_09
1527446	1527447	1	rd	2	rd	NC2752_09
1527938	1527939	1	rd	2	rd	NC2752_09
1531373	1531374	1	rd	2	rd	NC2752_09
1532568	1532569	1	rd	2	rd	NC2752_09
1534163	1534164	1	rd	2	rd	NC2752_09
1535172	1535173	1	rd	2	rd	NC2752_09
1536444	1536445	1	rd	2	rd	NC2752_09
1536778	1536779	1	rd	2	rd	NC2752_09
1537075	1537076	1	rd	2	rd	NC2752_09
1537783	1537784	1	rd	2	rd	NC2752_09
1538818	1538819	1	rd	2	rd	NC2752_09
1540655	1540656	1	rd	2	rd	NC2752_09
1541178	1541179	1	rd	2	rd	NC2752_09
1541951	1541952	44	rd	2	rd	NC2752_09
1543999	1544000	1	rd	2	rd	NC2752_09
1544304	1544305	1	rd	2	rd	NC2752_09
1546998	1546999	2	rd	2	rd	NC2752_09
1547088	1547089	1	rd	2	rd	NC2752_09
1547572	1547573	1	rd	2	rd	NC2752_09
1549695	1549696	1	rd	2	rd	NC2752_09
1552564	1552565	1	rd	2	rd	NC2752_09
1553593	1553594	1	rd	2	rd	NC2752_09
1553628	1553629	1	rd	2	rd	NC2752_09
1555103	1555104	2	rd	2	rd	NC2752_09
1555184	1555185	1	rd	2	rd	NC2752_09
1555511	1555512	3	rd	2	rd	NC2752_09
1555733	1555734	1	rd	2	rd	NC2752_09
1555788	1555789	1	rd	2	rd	NC2752_09
1556186	1556187	1	rd	2	rd	NC2752_09
1558945	1558946	1	rd	2	rd	NC2752_09
1559999	1560000	1	rd	2	rd	NC2752_09
1560394	1560395	2	rd	2	rd	NC2752_09
1561256	1561257	1	rd	2	rd	NC2752_09
1562622	1562623	1	rd	2	rd	NC2752_09
1563295	1563296	1	rd	2	rd	NC2752_09
1566272	1566273	1	rd	2	rd	NC2752_09
1566833	1566834	2	rd	2	rd	NC2752_09
1568611	1568612	1	rd	2	rd	NC2752_09
1570114	1570115	1	rd	2	rd	NC2752_09
1570913	1570914	1	rd	2	rd	NC2752_09
1571333	1571334	1	rd	2	rd	NC2752_09
1571884	1571885	1	rd	2	rd	NC2752_09
1572881	1572882	13	rd	2	rd	NC2752_09
1572878	1572879	1	rd	2	rd	NC2752_09
1573000	1573001	34	rd	2	rd	NC2752_09
1573000	1573001	18	rd	2	rd	NC2752_09
1573166	1573167	1	rd	2	rd	NC2752_09
1573318	1573319	1	rd	2	rd	NC2752_09
1573321	1573322	1	rd	2	rd	NC2752_09
1574045	1574046	1	rd	2	rd	NC2752_09
1574995	1574996	1	rd	2	rd	NC2752_09
1576636	1576637	1	rd	2	rd	NC2752_09
1576853	1576854	2	rd	2	rd	NC2752_09
1576998	1576999	1	rd	2	rd	NC2752_09
1577955	1577956	1	rd	2	rd	NC2752_09
1581163	1581164	1	rd	2	rd	NC2752_09
1581489	1581490	1	rd	2	rd	NC2752_09
						Total

Figure 7.6 (cont)

Breakpoints		Size (bp)		SCG	Isolate
Start	End	MRU-WTR Cluster			
1581696	1581697	1	rd	1	IGB423
1582068	1582070	1	NC	2	ARS7886
1583945	1583946	1	rd	2	CC35079
1584871	1584873	1	rd	2	NC2562_09
1587553	1587552	1	rd	2	NC2562_09
1587943	1587946	1	rd	2	NC2562_09
1592341	1592342	1	rd	2	NC2562_09
1594483	1594484	1	rd	2	NC2562_09
1594494	1594495	2	rd	2	NC2562_09
1596698	1596697	1	rd	2	NC2562_09
1597083	1597088	1	rd	2	NC2562_09
1597128	1597130	2	rd	2	NC2562_09
1598274	1598275	1	rd	2	NC2562_09
1598323	1598326	1	rd	2	NC2562_09
1600834	1600835	2	rd	2	NC2562_09
1601521	1601522	1	rd	2	NC2562_09
1601711	1602132	1	rd	2	NC2562_09
1603999	1604000	1	rd	2	NC2562_09
1604598	1604599	1	rd	2	NC2562_09
1609999	1610000	1	rd	2	NC2562_09
1612271	1612272	6	rd	2	NC2562_09
1612372	1612373	1	rd	2	NC2562_09
1612624	1612625	21	rd	2	NC2562_09
1612627	1612628	42	rd	2	NC2562_09
1615078	1615079	1	rd	2	NC2562_09
1615999	1616000	1	rd	2	NC2562_09
1617588	1617587	1	rd	2	NC2562_09
1618303	1618303	1	rd	2	NC2562_09
1618733	1618732	16	rd	2	NC2562_09
1618818	1618819	1	rd	2	NC2562_09
1620700	1620701	1	rd	2	NC2562_09
1620762	1620764	1	rd	2	NC2562_09
1621559	1621560	2	rd	2	NC2562_09
1621613	1621613	1	rd	2	NC2562_09
1623538	1623539	1	rd	2	NC2562_09
1625333	1625333	3	rd	2	NC2562_09
1625342	1625348	1	rd	2	NC2562_09
1626764	1626765	1	rd	2	NC2562_09
1626872	1626873	1	rd	2	NC2562_09
1627118	1627119	1	rd	2	NC2562_09
1627155	1627156	1	rd	2	NC2562_09
1628253	1628254	1	rd	2	NC2562_09
1628973	1628980	1	rd	2	NC2562_09
1629973	1629974	1	rd	2	NC2562_09
1630784	1630785	2	rd	2	NC2562_09
1631041	1631048	1	rd	2	NC2562_09
1631299	1631300	1	rd	2	NC2562_09
1631464	1631465	8	rd	2	NC2562_09
1631748	1631749	14	rd	2	NC2562_09
1631846	1631847	15	rd	2	NC2562_09
1632149	1632150	9	rd	2	NC2562_09
1632509	1632508	1	rd	2	NC2562_09
1632511	1632511	27	rd	2	NC2562_09
1632631	1632632	8	rd	2	NC2562_09
1632824	1632825	8	rd	2	NC2562_09
1632941	1632944	1	rd	2	NC2562_09
1632966	1632966	1	rd	2	NC2562_09
1633094	1633095	1	rd	2	NC2562_09
1633729	1633730	1	rd	2	NC2562_09
1633813	1633816	5	rd	2	NC2562_09
1634075	1634076	2	rd	2	NC2562_09
1635611	1635614	1	rd	2	NC2562_09
1636172	1636173	3	rd	2	NC2562_09
1637627	1637628	2	rd	2	NC2562_09
1641638	1641637	1	rd	2	NC2562_09
1641853	1641852	1	rd	2	NC2562_09
1642999	1643000	1	rd	2	NC2562_09
1647759	1647760	3	rd	2	NC2562_09
1649999	1650000	2	rd	2	NC2562_09
1650530	1650533	1	rd	2	NC2562_09
1651292	1651293	1	rd	2	NC2562_09
1654454	1654455	1	rd	2	NC2562_09
1655678	1655677	1	rd	2	NC2562_09
1655723	1655726	1	rd	2	NC2562_09
1656016	1656017	1	rd	2	NC2562_09
1656353	1656354	1	rd	2	NC2562_09
1656463	1656464	1	rd	2	NC2562_09
1656609	1656610	1	rd	2	NC2562_09
1656640	1656647	1	rd	2	NC2562_09
1658831	1658834	1	rd	2	NC2562_09
1659146	1659147	1	rd	2	NC2562_09
1660311	1660312	1	rd	2	NC2562_09
1660548	1660550	1	rd	2	NC2562_09
1661099	1661100	1	rd	2	NC2562_09
1661545	1661550	1	rd	2	NC2562_09
1663528	1663529	1	rd	2	NC2562_09
1664217	1664218	2	rd	2	NC2562_09
1664963	1664966	2	rd	2	NC2562_09
1668191	1668192	1	rd	2	NC2562_09
1668376	1668377	1	rd	2	NC2562_09
1669993	1669995	1	rd	2	NC2562_09
1670510	1670511	1	rd	2	NC2562_09
1671032	1671033	1	rd	2	NC2562_09
1671608	1671609	1	rd	2	NC2562_09
1674179	1674180	1	rd	2	NC2562_09
1676095	1676096	1	rd	2	NC2562_09
1678045	1678050	1	rd	2	NC2562_09
1682038	1682039	1	rd	2	NC2562_09
1682084	1682086	1	rd	2	NC2562_09
1683784	1683785	2	rd	2	NC2562_09
1684728	1684729	1	rd	2	NC2562_09
1685568	1685569	1	rd	2	NC2562_09
1687766	1687768	1	rd	2	NC2562_09
1688999	1689000	1	rd	2	NC2562_09
1689030	1689031	1	rd	2	NC2562_09
1689665	1689666	3	rd	2	NC2562_09
1689901	1689904	1	rd	2	NC2562_09
1690733	1690734	1	rd	2	NC2562_09
1691810	1691811	1	rd	2	NC2562_09
1692181	1692182	1	rd	2	NC2562_09
1693853	1693854	1	rd	2	NC2562_09
1693984	1693985	1	rd	2	NC2562_09
1694265	1694266	2	rd	2	NC2562_09
1696898	1696899	1	rd	2	NC2562_09
1697478	1697479	1	rd	2	NC2562_09
1697532	1697533	2	rd	2	NC2562_09
1698154	1698155	1	rd	2	NC2562_09
1698377	1698378	1	rd	2	NC2562_09
1698753	1698752	1	rd	2	NC2562_09
1700061	1700066	1	rd	2	NC2562_09
1700714	1700717	2	rd	2	NC2562_09
1700914	1700917	1	rd	2	NC2562_09
1702361	1702368	1	rd	2	NC2562_09
1705472	1705477	1	rd	2	NC2562_09
1705568	1705569	1	rd	2	NC2562_09
1708416	1708417	1	rd	2	NC2562_09
1708472	1708473	1	rd	2	NC2562_09
1708938	1708939	1	rd	2	NC2562_09
1708958	1708959	1	rd	2	NC2562_09
1710251	1710258	1	rd	2	NC2562_09
1711216	1711217	1	rd	2	NC2562_09
1713853	1713854	1	rd	2	NC2562_09
1715430	1715433	1	rd	2	NC2562_09
1715488	1715490	1	rd	2	NC2562_09
1715545	1715546	1	rd	2	NC2562_09
1715838	1715839	1	rd	2	NC2562_09
1716342	1716345	1	rd	2	NC2562_09
1717453	1717458	1	rd	2	NC2562_09
1717764	1717765	1	rd	2	NC2562_09
1722616	1722617	1	rd	2	NC2562_09
1722721	1722728	1	rd	2	NC2562_09
1723721	1723728	1	rd	2	NC2562_09
1725445	1725446	1	rd	2	NC2562_09
1728296	1728299	1	rd	2	NC2562_09
1731334	1731335	1	rd	2	NC2562_09
1731971	1731972	2	rd	2	NC2562_09
1731978	1731979	2	rd	2	NC2562_09
1732271	1732278	1	rd	2	NC2562_09
1734865	1734866	1	rd	2	NC2562_09
1735346	1735347	1	rd	2	NC2562_09
1737053	1737052	6	rd	2	NC2562_09
1738392	1738393	1	rd	2	NC2562_09
Total				18	

Breakpoints		Size (bp)	MRU-WTR Cluster	SCG	Isolate
Start	End				
1739102	1739108	1	rd	1	RGB423
1739944	1739945	2	NC	2	ARS7886
1739972	1739972	1	rd	2	CC35079
1742128	1742129	1	rd	2	NC256_09
1743098	1743099	1	rd	2	NC256_09
1743161	1743162	1	rd	2	NC256_09
1743304	1743305	2	rd	2	NC256_09
1745413	1745414	1	rd	2	NC256_09
1746455	1746456	1	rd	2	NC256_09
1746931	1746932	1	rd	2	NC256_09
1747093	1747098	2	rd	2	NC256_09
1749543	1749542	2	rd	2	NC256_09
1749998	1749999	2	rd	2	NC256_09
1753472	1753473	1	rd	2	NC256_09
1753515	1753520	1	rd	2	NC256_09
1754016	1754017	1	rd	2	NC256_09
1755281	1755288	2	rd	2	NC256_09
1756181	1756182	1	rd	2	NC256_09
1756286	1756287	1	rd	2	NC256_09
1757943	1757946	1	rd	2	NC256_09
1757998	1757999	1	rd	2	NC256_09
1758745	1758746	1	rd	2	NC256_09
1759111	1759112	1	rd	2	NC256_09
1760661	1760662	1	rd	2	NC256_09
1762993	1762994	1	rd	2	NC256_09
1768498	1768499	1	rd	2	NC256_09
1769228	1769229	1	rd	2	NC256_09
1770671	1770672	1	rd	2	NC256_09
1770999	1771000	1	rd	2	NC256_09
1771128	1771129	1	rd	2	NC256_09
1773442	1773443	1	rd	2	NC256_09
1774645	1774646	1	rd	2	NC256_09
1778942	1778943	1	rd	2	NC256_09
1779953	1779954	1	rd	2	NC256_09
1780598	1780599	1	rd	2	NC256_09
1780612	1780613	1	rd	2	NC256_09
1781529	1781530	1	rd	2	NC256_09
1781585	1781586	1	rd	2	NC256_09
1781724	1781725	1	rd	2	NC256_09
1782748	1782749	1	rd	2	NC256_09
1784066	1784067	1	rd	2	NC256_09
1789193	1789194	1	rd	2	NC256_09
1790656	1790657	1	rd	2	NC256_09
1791611	1791612	1	rd	2	NC256_09
1791154	1791155	1	rd	2	NC256_09
1794433	1794434	1	rd	2	NC256_09
1795764	1795765	1	rd	2	NC256_09
1795854	1795855	1	rd	2	NC256_09
1795906	1795907	1	rd	2	NC256_09
1797676	1797677	1	rd	2	NC256_09
1798393	1798394	1	rd	2	NC256_09
1800316	1800317	2	rd	2	NC256_09
1800381	1800382	1	rd	2	NC256_09
1802348	1802349	1	rd	2	NC256_09
1802431	1802432	1	rd	2	NC256_09
1802481	1802482	1	rd	2	NC256_09
1802869	1802870	1	rd	2	NC256_09
1803316	1803317	1	rd	2	NC256_09
1805656	1805657	1	rd	2	NC256_09
1811052	1811053	2	rd	2	NC256_09
1811693	1811694	1	rd	2	NC256_09
1812753	1812754	2	rd	2	NC256_09
1812999	1813000	1	rd	2	NC256_09
1814343	1814344	1	rd	2	NC256_09
1814456	1814457	1	rd	2	NC256_09
1815688	1815689	1	rd	2	NC256_09
1820147	1820148	1	rd	2	NC256_09
1822728	1822729	2	rd	2	NC256_09
1823039	1823040	1	rd	2	NC256_09
1824386	1824387	1	rd	2	NC256_09
1824434	1824435	1	rd	2	NC256_09
1824684	1824685	1	rd	2	NC256_09
1824998	1824999	1	rd	2	NC256_09
1825476	1825477	1	rd	2	NC256_09
1825830	1825831	3	rd	2	NC256_09
1827146	1827147	1	rd	2	NC256_09
1827470	1827471	1	rd	2	NC256_09
1827923	1827924	1	rd	2	NC256_09
1828497	1828498	1	rd	2	NC256_09
1829738	1829739	1	rd	2	NC256_09
1831079	1831080	1	rd	2	NC256_09
1833359	1833360	1	rd	2	NC256_09
1833544	1833545	1	rd	2	NC256_09
1833714	1833715	1	rd	2	NC256_09
1834204	1834205	1	rd	2	NC256_09
1834520	1834521	2	rd	2	NC256_09
1838998	1838999	1	rd	2	NC256_09
1839687	1839688	2	rd	2	NC256_09
1840338	1840339	1	rd	2	NC256_09
1841753	1841754	2	rd	2	NC256_09
1841778	1841779	1	rd	2	NC256_09
1843166	1843167	1	rd	2	NC256_09
1843570	1843571	37	rd	2	NC256_09
1845593	1845594	57	rd	2	NC256_09
1846424	1846425	1	rd	2	NC256_09
1847305	1847306	1	rd	2	NC256_09
1849082	1849083	1	rd	2	NC256_09
1851898	1851899	1	rd	2	NC256_09
1851938	1851939	2	rd	2	NC256_09
1852933	1852934	1	rd	2	NC256_09
1854540	1854541	2	rd	2	NC256_09
1854674	1854675	1	rd	2	NC256_09
1857788	1857789	1	rd	2	NC256_09
1858483	1858484	1	rd	2	NC256_09
1860298	1860299	1	rd	2	NC256_09
1861478	1861479	1	rd	2	NC256_09
1861819	1861820	3	rd	2	NC256_09
1862922	1862923	1	rd	2	NC256_09
1863366	1863367	1	rd	2	NC256_09
1864708	1864709	21	rd	2	NC256_09
1864924	1864925	21	rd	2	NC256_09
1864930	1864931	1	rd	2	NC256_09
1865116	1865117	1	rd	2	NC256_09
1866269	1866270	3	rd	2	NC256_09
1866322	1866323	1	rd	2	NC256_09
1866465	1866466	1	rd	2	NC256_09
1866807	1866808	1	rd	2	NC256_09
1871753	1871754	2	rd	2	NC256_09
1871803	1871804	1	rd	2	NC256_09
1873606	1873607	2	rd	2	NC256_09
1874117	1874118	1	rd	2	NC256_09
1875623	1875624	1	rd	2	NC256_09
1879963	1879964	1	rd	2	NC256_09
1881018	1881019	1	rd	2	NC256_09
1881684	1881685	1	rd	2	NC256_09
1882598	1882599	1	rd	2	NC256_09
1884110	1884111	22	rd	2	NC256_09
1884187	1884188	2	rd	2	NC256_09
1884343	1884344	1	rd	2	NC256_09
1884915	1884916	3	rd	2	NC256_09
1884953	1884954	2	rd	2	NC256_09
1884962	1884963	2	rd	2	NC256_09
1884998	1884999	2	rd	2	NC256_09
1885412	1885413	2	rd	2	NC256_09
1887054	1887055	1	rd	2	NC256_09
1887293	1887294	1	rd	2	NC256_09
1887511	1887512	1	rd	2	NC256_09
1889645	1889646	1	rd	2	NC256_09
1889698	1889699	1	rd	2	NC256_09
1889998	1889999	1	rd	2	NC256_09
1890072	1890073	1	rd	2	NC256_09
1890254	1890255	1	rd	2	NC256_09
1890305	1890306	2	rd	2	NC256_09
1891347	1891348	1	rd	2	NC256_09
1893851	1893852	1	rd	2	NC256_09
1893433	1893434	1	rd	2	NC256_09
1893488	1893489	2	rd	2	NC256_09
1894300	1894301	10	rd	2	NC256_09
1894344	1894345	1	rd	2	NC256_09
1896943	1896944	2	rd	2	NC256_09
1899989	1899990	3	rd	2	NC256_09
Total					70

Figure 7.6 (cont)

Breakpoints		Size (bp)		SCG	Isolate
Start	End	MRU-WTR Cluster			
1899998	1899997	rd	1	1	IGTB423
1900433	1900434	rd	1	1	ARS7886
1901172	1901178	rd	1	1	CC35079
1901281	1901388	rd	1	1	NC2502_09
1901968	1901969	rd	1	1	NC2502_09
1901998	1901999	rd	1	1	NC2502_09
1901999	1902000	rd	1	1	NC2502_09
1902243	1902244	rd	1	1	NC2502_09
1902522	1902523	rd	1	1	NC2502_09
1904433	1904434	rd	1	1	NC2502_09
1904484	1904485	rd	1	1	NC2502_09
1907973	1907974	rd	1	1	NC2502_09
1909444	1909445	rd	1	1	NC2502_09
1909522	1909523	rd	1	1	NC2502_09
1909692	1909693	rd	1	1	NC2502_09
1915122	1915123	rd	1	1	NC2502_09
1918098	1918099	rd	1	1	NC2502_09
1919133	1919134	rd	1	1	NC2502_09
1919908	1919909	rd	1	1	NC2502_09
1920446	1920447	rd	1	1	NC2502_09
1920495	1920496	rd	1	1	NC2502_09
1920853	1920854	rd	1	1	NC2502_09
1923438	1923439	rd	1	1	NC2502_09
1923655	1923656	rd	1	1	NC2502_09
1924155	1924156	rd	1	1	NC2502_09
1926250	1926251	rd	1	1	NC2502_09
1927388	1927389	rd	1	1	NC2502_09
1928388	1928389	rd	1	1	NC2502_09
1929955	1929956	rd	1	1	NC2502_09
1931101	1931102	rd	1	1	NC2502_09
1932672	1932673	rd	1	1	NC2502_09
1938622	1938623	rd	1	1	NC2502_09
1938413	1938414	rd	1	1	NC2502_09
1938462	1938463	rd	1	1	NC2502_09
1938518	1938519	rd	1	1	NC2502_09
1943083	1943084	rd	1	1	NC2502_09
1943822	1943823	rd	1	1	NC2502_09
1944464	1944465	rd	1	1	NC2502_09
1946538	1946539	rd	1	1	NC2502_09
1947020	1947021	rd	1	1	NC2502_09
1948998	1948999	rd	1	1	NC2502_09
1951522	1951523	rd	1	1	NC2502_09
1951974	1951975	rd	1	1	NC2502_09
1952038	1952039	rd	1	1	NC2502_09
1952198	1952199	rd	1	1	NC2502_09
1952252	1952253	rd	1	1	NC2502_09
1953742	1953743	rd	1	1	NC2502_09
1953998	1953999	rd	1	1	NC2502_09
1954186	1954187	rd	1	1	NC2502_09
1954499	1954500	rd	1	1	NC2502_09
1955596	1955597	rd	1	1	NC2502_09
1955611	1955612	rd	1	1	NC2502_09
1956114	1956115	rd	1	1	NC2502_09
1959088	1959089	rd	1	1	NC2502_09
1959784	1959785	rd	1	1	NC2502_09
1959953	1959954	rd	1	1	NC2502_09
1960725	1960726	rd	1	1	NC2502_09
1960755	1960756	rd	1	1	NC2502_09
1961488	1961489	rd	1	1	NC2502_09
1962788	1962789	rd	1	1	NC2502_09
1963723	1963724	rd	1	1	NC2502_09
1963776	1963777	rd	1	1	NC2502_09
1965111	1965112	rd	1	1	NC2502_09
1966372	1966373	rd	1	1	NC2502_09
1971103	1971104	rd	1	1	NC2502_09
1971152	1971153	rd	1	1	NC2502_09
1972128	1972129	rd	1	1	NC2502_09
1972242	1972243	rd	1	1	NC2502_09
1973033	1973034	rd	1	1	NC2502_09
1974188	1974189	rd	1	1	NC2502_09
1974264	1974265	rd	1	1	NC2502_09
1976666	1976667	rd	1	1	NC2502_09
1980454	1980455	rd	1	1	NC2502_09
1983698	1983699	rd	1	1	NC2502_09
1983773	1983774	rd	1	1	NC2502_09
1986699	1986700	rd	1	1	NC2502_09
1990596	1990597	rd	1	1	NC2502_09
1990716	1990717	rd	1	1	NC2502_09
1991653	1991654	rd	1	1	NC2502_09
1991809	1991810	rd	1	1	NC2502_09
1992198	1992199	rd	1	1	NC2502_09
1992528	1992529	rd	1	1	NC2502_09
1993118	1993119	rd	1	1	NC2502_09
1993645	1993646	rd	1	1	NC2502_09
1994305	1994306	rd	1	1	NC2502_09
1994552	1994553	rd	1	1	NC2502_09
1994558	1994559	rd	1	1	NC2502_09
1994597	1994598	rd	1	1	NC2502_09
1999133	1999134	rd	1	1	NC2502_09
1999658	1999659	rd	1	1	NC2502_09
1999923	1999924	rd	1	1	NC2502_09
2000033	2000034	rd	1	1	NC2502_09
2000575	2000576	rd	1	1	NC2502_09
2002712	2002713	rd	1	1	NC2502_09
2003504	2003505	rd	1	1	NC2502_09
2004666	2004667	rd	1	1	NC2502_09
2006201	2006202	rd	1	1	NC2502_09
2009130	2009131	rd	1	1	NC2502_09
2009533	2009534	rd	1	1	NC2502_09
2009688	2009689	rd	1	1	NC2502_09
2009774	2009775	rd	1	1	NC2502_09
2013145	2013146	rd	1	1	NC2502_09
2013991	2013992	rd	1	1	NC2502_09
2016133	2016134	rd	1	1	NC2502_09
2016163	2016164	rd	1	1	NC2502_09
2017514	2017515	rd	1	1	NC2502_09
2019315	2019316	rd	1	1	NC2502_09
2023088	2023089	rd	1	1	NC2502_09
2024912	2024913	rd	1	1	NC2502_09
2027738	2027739	rd	1	1	NC2502_09
2029638	2029639	rd	1	1	NC2502_09
2032068	2032069	rd	1	1	NC2502_09
2032884	2032885	rd	1	1	NC2502_09
2033446	2033447	rd	1	1	NC2502_09
2033733	2033734	rd	1	1	NC2502_09
2036645	2036646	rd	1	1	NC2502_09
2041028	2041029	rd	1	1	NC2502_09
2042048	2042049	rd	1	1	NC2502_09
2044240	2044241	rd	1	1	NC2502_09
2044355	2044356	rd	1	1	NC2502_09
2049774	2049775	rd	1	1	NC2502_09
2050513	2050514	rd	1	1	NC2502_09
2050773	2050774	rd	1	1	NC2502_09
2050786	2050787	rd	1	1	NC2502_09
2054678	2054679	rd	1	1	NC2502_09
2057753	2057754	rd	1	1	NC2502_09
2058315	2058316	rd	1	1	NC2502_09
2058646	2058647	rd	1	1	NC2502_09
2060398	2060399	rd	1	1	NC2502_09
2061433	2061434	rd	1	1	NC2502_09
2061528	2061529	rd	1	1	NC2502_09
2061938	2061939	rd	1	1	NC2502_09
2061958	2061959	rd	1	1	NC2502_09
2063975	2063976	rd	1	1	NC2502_09
2063594	2063595	rd	1	1	NC2502_09
2067394	2067395	rd	1	1	NC2502_09
2067908	2067909	rd	1	1	NC2502_09
2069913	2069914	rd	1	1	NC2502_09
2070223	2070224	rd	1	1	NC2502_09
2070924	2070925	rd	1	1	NC2502_09
2072124	2072125	rd	1	1	NC2502_09
2072413	2072414	rd	1	1	NC2502_09
2072783	2072784	rd	1	1	NC2502_09
2073346	2073347	rd	1	1	NC2502_09
2076425	2076426	rd	1	1	NC2502_09
2076968	2076969	rd	1	1	NC2502_09
2080871	2080872	rd	1	1	NC2502_09
2081153	2081154	rd	1	1	NC2502_09
2081449	2081450	rd	1	1	NC2502_09
2087584	2087585	rd	1	1	NC2502_09
2087748	2087749	rd	1	1	NC2502_09
Total				18	



Figure 7.6 (cont)

Breakpoints		Size (bp)	SCG	Isolate
Start	End		MRU-WTR Cluster	
2088046	2088047	1	rd	IGTB423
2089011	2089018	1	NC	ARS7886
2089248	2089258	1	NC	CC35079
2089484	2089485	1	rd	CC250_09
2089778	2089780	1	NC	NC250_09
2091228	2091229	1	rd	NC250_09
2093451	2093452	1	rd	NC250_09
2096388	2096387	1	rd	NC250_09
2097962	2097963	1	rd	NC250_09
2100514	2100515	1	rd	NC250_09
2100517	2100517	1	rd	NC250_09
2102103	2102104	1	rd	NC250_09
2102112	2102111	2	rd	NC250_09
2103210	2103211	1	rd	NC250_09
2103256	2103257	1	rd	NC250_09
2107506	2107507	2	rd	NC250_09
2109524	2109524	1	rd	NC250_09
2112241	2112248	1	rd	NC250_09
2112295	2112296	1	rd	NC250_09
2112870	2112871	1	rd	NC250_09
2112954	2112955	1	rd	NC250_09
2116778	2116779	1	rd	NC250_09
2117301	2117304	1	rd	NC250_09
2121578	2121579	1	rd	NC250_09
2121445	2121450	1	rd	NC250_09
2124045	2124046	1	rd	NC250_09
2125531	2125538	1	rd	NC250_09
2125698	2125699	1	rd	NC250_09
2125758	2125759	1	rd	NC250_09
2127354	2127355	1	rd	NC250_09
2130775	2130786	1	rd	NC250_09
2132355	2132360	1	rd	NC250_09
2133468	2133469	15	rd	NC250_09
2133478	2133479	15	rd	NC250_09
2133481	2133488	1	rd	NC250_09
2136338	2136337	2	rd	NC250_09
2136391	2136392	1	rd	NC250_09
2136999	2137000	1	rd	NC250_09
2137521	2137522	1	rd	NC250_09
2137521	2137522	10	rd	NC250_09
2137555	2137556	1	rd	NC250_09
2137621	2137622	1	rd	NC250_09
2138881	2138888	1	rd	NC250_09
2141186	2141187	1	rd	NC250_09
2141469	2141470	1	rd	NC250_09
2141771	2141772	2	rd	NC250_09
2142750	2142750	2	rd	NC250_09
2142812	2142813	1	rd	NC250_09
2142866	2142867	1	rd	NC250_09
2143128	2143127	1	rd	NC250_09
2143430	2143440	1	rd	NC250_09
2143521	2143524	1	rd	NC250_09
2146051	2146060	45	rd	NC250_09
2148221	2148222	1	rd	NC250_09
2148571	2148574	1	rd	NC250_09
2148653	2148658	1	rd	NC250_09
2152841	2152843	3	rd	NC250_09
2155327	2155328	2	rd	NC250_09
2155985	2155986	1	rd	NC250_09
2156113	2156118	1	rd	NC250_09
2159741	2159742	2	rd	NC250_09
2161343	2161344	1	rd	NC250_09
2161684	2161688	1	rd	NC250_09
2164728	2164729	1	rd	NC250_09
2165911	2165914	72	rd	NC250_09
2165916	2165917	72	rd	NC250_09
2165924	2165925	72	rd	NC250_09
2165925	2165930	72	rd	NC250_09
2165933	2165934	71	rd	NC250_09
2167388	2167389	1	rd	NC250_09
2171482	2171483	1	rd	NC250_09
2172888	2172890	1	rd	NC250_09
2175215	2175216	1	rd	NC250_09
2175914	2175915	1	rd	NC250_09
2176144	2176145	2	rd	NC250_09
2177586	2177587	1	rd	NC250_09
2179606	2179607	1	rd	NC250_09
2179771	2179772	2	rd	NC250_09
2181340	2181340	1	rd	NC250_09
2182326	2182327	1	rd	NC250_09
2189705	2189706	2	rd	NC250_09
2192116	2192117	1	rd	NC250_09
2192284	2192285	1	rd	NC250_09
2193841	2193842	2	rd	NC250_09
2195551	2195558	1	rd	NC250_09
2196850	2196860	44	rd	NC250_09
2196881	2196882	28	rd	NC250_09
2197253	2197254	1	rd	NC250_09
2201286	2201289	1	rd	NC250_09
2201702	2201703	1	rd	NC250_09
2204310	2204311	1	rd	NC250_09
2204378	2204377	1	rd	NC250_09
2204434	2204435	1	rd	NC250_09
2205628	2205629	1	rd	NC250_09
2205764	2205765	2	rd	NC250_09
2205811	2205812	2	rd	NC250_09
2206105	2206106	2	rd	NC250_09
2206161	2206168	1	rd	NC250_09
2206723	2206724	1	rd	NC250_09
2207591	2207593	1	rd	NC250_09
2208561	2208562	1	rd	NC250_09
2208911	2208914	2	rd	NC250_09
2209651	2209652	1	rd	NC250_09
2212272	2212273	1	rd	NC250_09
2213861	2213862	2	rd	NC250_09
2215385	2215386	1	rd	NC250_09
2216381	2216383	1	rd	NC250_09
2218521	2218522	2	rd	NC250_09
2219806	2219807	1	rd	NC250_09
2221320	2221318	1	rd	NC250_09
2223548	2223550	1	rd	NC250_09
2223509	2223510	2	rd	NC250_09
2225786	2225787	1	rd	NC250_09
2228441	2228445	2	rd	NC250_09
2229141	2229142	1	rd	NC250_09
2229671	2229676	1	rd	NC250_09
2230291	2230292	2	rd	NC250_09
2233758	2233759	1	rd	NC250_09
2234241	2234246	1	rd	NC250_09
2234241	2234246	2	rd	NC250_09
2235326	2235327	1	rd	NC250_09
2235576	2235577	1	rd	NC250_09
2236796	2236797	1	rd	NC250_09
2239046	2239045	1	rd	NC250_09
2239061	2239062	1	rd	NC250_09
2242750	2242751	1	rd	NC250_09
2243191	2243194	1	rd	NC250_09
2243679	2243680	1	rd	NC250_09
2243730	2243731	1	rd	NC250_09
2243778	2243777	2	rd	NC250_09
2244865	2244870	1	rd	NC250_09
2245424	2245425	1	rd	NC250_09
2248480	2248481	1	rd	NC250_09
2248511	2248518	1	rd	NC250_09
2248886	2248888	1	rd	NC250_09
2249421	2249422	1	rd	NC250_09
2249474	2249475	1	rd	NC250_09
2253664	2253665	1	rd	NC250_09
2253408	2253407	1	rd	NC250_09
2253999	2254000	1	rd	NC250_09
2254555	2254556	1	rd	NC250_09
2255115	2255110	1	rd	NC250_09
2256703	2256704	1	rd	NC250_09
2257571	2257574	2	rd	NC250_09
2258861	2258863	1	rd	NC250_09
2260146	2260147	1	rd	NC250_09
2260839	2260840	1	rd	NC250_09
2261311	2261312	1	rd	NC250_09
2264088	2264089	1	rd	NC250_09
2269131	2269134	1	rd	NC250_09
2270525	2270530	1	rd	NC250_09
2270911	2270914	1	rd	NC250_09
			Total	

Breakpoints		Size (bp)		SCG	Isolate
Start	End	MRU-WTR Cluster	SCG		
		rd	1	NC	NC
		rd	2	AS5196	AS5196
		rd	3	AS5197	AS5197
		rd	4	AS5198	AS5198
		rd	5	AS5199	AS5199
		rd	6	AS5200	AS5200
		rd	7	AS5201	AS5201
		rd	8	AS5202	AS5202
		rd	9	AS5203	AS5203
		rd	10	AS5204	AS5204
		rd	11	AS5205	AS5205
		rd	12	AS5206	AS5206
		rd	13	AS5207	AS5207
		rd	14	AS5208	AS5208
		rd	15	AS5209	AS5209
		rd	16	AS5210	AS5210
		rd	17	AS5211	AS5211
		rd	18	AS5212	AS5212
		rd	19	AS5213	AS5213
		rd	20	AS5214	AS5214
		rd	21	AS5215	AS5215
		rd	22	AS5216	AS5216
		rd	23	AS5217	AS5217
		rd	24	AS5218	AS5218
		rd	25	AS5219	AS5219
		rd	26	AS5220	AS5220
		rd	27	AS5221	AS5221
		rd	28	AS5222	AS5222
		rd	29	AS5223	AS5223
		rd	30	AS5224	AS5224
		rd	31	AS5225	AS5225
		rd	32	AS5226	AS5226
		rd	33	AS5227	AS5227
		rd	34	AS5228	AS5228
		rd	35	AS5229	AS5229
		rd	36	AS5230	AS5230
		rd	37	AS5231	AS5231
		rd	38	AS5232	AS5232
		rd	39	AS5233	AS5233
		rd	40	AS5234	AS5234
		rd	41	AS5235	AS5235
		rd	42	AS5236	AS5236
		rd	43	AS5237	AS5237
		rd	44	AS5238	AS5238
		rd	45	AS5239	AS5239
		rd	46	AS5240	AS5240
		rd	47	AS5241	AS5241
		rd	48	AS5242	AS5242
		rd	49	AS5243	AS5243
		rd	50	AS5244	AS5244
		rd	51	AS5245	AS5245
		rd	52	AS5246	AS5246
		rd	53	AS5247	AS5247
		rd	54	AS5248	AS5248
		rd	55	AS5249	AS5249
		rd	56	AS5250	AS5250
		rd	57	AS5251	AS5251
		rd	58	AS5252	AS5252
		rd	59	AS5253	AS5253
		rd	60	AS5254	AS5254
		rd	61	AS5255	AS5255
		rd	62	AS5256	AS5256
		rd	63	AS5257	AS5257
		rd	64	AS5258	AS5258
		rd	65	AS5259	AS5259
		rd	66	AS5260	AS5260
		rd	67	AS5261	AS5261
		rd	68	AS5262	AS5262
		rd	69	AS5263	AS5263
		rd	70	AS5264	AS5264
		rd	71	AS5265	AS5265
		rd	72	AS5266	AS5266
		rd	73	AS5267	AS5267
		rd	74	AS5268	AS5268
		rd	75	AS5269	AS5269
		rd	76	AS5270	AS5270
		rd	77	AS5271	AS5271
		rd	78	AS5272	AS5272
		rd	79	AS5273	AS5273
		rd	80	AS5274	AS5274
		rd	81	AS5275	AS5275
		rd	82	AS5276	AS5276
		rd	83	AS5277	AS5277
		rd	84	AS5278	AS5278
		rd	85	AS5279	AS5279
		rd	86	AS5280	AS5280
		rd	87	AS5281	AS5281
		rd	88	AS5282	AS5282
		rd	89	AS5283	AS5283
		rd	90	AS5284	AS5284
		rd	91	AS5285	AS5285
		rd	92	AS5286	AS5286
		rd	93	AS5287	AS5287
		rd	94	AS5288	AS5288
		rd	95	AS5289	AS5289
		rd	96	AS5290	AS5290
		rd	97	AS5291	AS5291
		rd	98	AS5292	AS5292
		rd	99	AS5293	AS5293
		rd	100	AS5294	AS5294
		rd	101	AS5295	AS5295
		rd	102	AS5296	AS5296
		rd	103	AS5297	AS5297
		rd	104	AS5298	AS5298
		rd	105	AS5299	AS5299
		rd	106	AS5300	AS5300
		rd	107	AS5301	AS5301
		rd	108	AS5302	AS5302
		rd	109	AS5303	AS5303
		rd	110	AS5304	AS5304
		rd	111	AS5305	AS5305
		rd	112	AS5306	AS5306
		rd	113	AS5307	AS5307
		rd	114	AS5308	AS5308
		rd	115	AS5309	AS5309
		rd	116	AS5310	AS5310
		rd	117	AS5311	AS5311
		rd	118	AS5312	AS5312
		rd	119	AS5313	AS5313
		rd	120	AS5314	AS5314
		rd	121	AS5315	AS5315
		rd	122	AS5316	AS5316
		rd	123	AS5317	AS5317
		rd	124	AS5318	AS5318
		rd	125	AS5319	AS5319
		rd	126	AS5320	AS5320
		rd	127	AS5321	AS5321
		rd	128	AS5322	AS5322
		rd	129	AS5323	AS5323
		rd	130	AS5324	AS5324
		rd	131	AS5325	AS5325
		rd	132	AS5326	AS5326
		rd	133	AS5327	AS5327
		rd	134	AS5328	AS5328
		rd	135	AS5329	AS5329
		rd	136	AS5330	AS5330
		rd	137	AS5331	AS5331
		rd	138	AS5332	AS5332
		rd	139	AS5333	AS5333
		rd	140	AS5334	AS5334
		rd	141	AS5335	AS5335
		rd	142	AS5336	AS5336
		rd	143	AS5337	AS5337
		rd	144	AS5338	AS5338
		rd	145	AS5339	AS5339
		rd	146	AS5340	AS5340
		rd	147	AS5341	AS5341
		rd	148	AS5342	AS5342
		rd	149	AS5343	AS5343
		rd	150	AS5344	AS5344
		rd	151	AS5345	AS5345
		rd	152	AS5346	AS5346
		rd	153	AS5347	AS5347
		rd	154	AS5348	AS5348
		rd	155	AS5349	AS5349
		rd	156	AS5350	AS5350
		rd	157	AS5351	AS5351
		rd	158	AS5352	AS5352
		rd	159	AS5353	AS5353
		rd	160	AS5354	AS5354
		rd	161	AS5355	AS5355
		rd	162	AS5356	AS5356
		rd	163	AS5357	AS5357
		rd	164	AS5358	AS5358
		rd	165	AS5359	AS5359
		rd	166	AS5360	AS5360
		rd	167	AS5361	AS5361
		rd	168	AS5362	AS5362
		rd	169	AS5363	AS5363
		rd	170	AS5364	AS5364
		rd	171	AS5365	AS5365
		rd	172	AS5366	AS5366
		rd	173	AS5367	AS5367
		rd	174	AS5368	AS5368
		rd	175	AS5369	AS5369
		rd	176	AS5370	AS5370
		rd	177	AS5371	AS5371
		rd	178	AS5372	AS5372
		rd	179	AS5373	AS5373
		rd	180	AS5374	AS5374
		rd	181	AS5375	AS5375
		rd	182	AS5376	AS5376
		rd	183	AS5377	AS5377
		rd	184	AS5378	AS5378
		rd	185	AS5379	AS5379
		rd	186	AS5380	AS5380
		rd	187	AS5381	AS5381
		rd	188	AS5382	AS5382
		rd	189	AS5383	AS5383
		rd	190	AS5384	AS5384
		rd	191	AS5385	AS5385
		rd	192	AS5386	AS5386
		rd	193	AS5387	AS5387
		rd	194	AS5388	AS5388
		rd	195	AS5389	AS5389
		rd	196	AS5390	AS5390
		rd	197	AS5391	AS5391
		rd	198	AS5392	AS5392
		rd	199	AS5393	AS5393
		rd	200	AS5394	AS5394
		rd	201	AS5395	AS5395
		rd	202	AS5396	AS5396
		rd	203	AS5397	AS5397
		rd	204	AS5398	AS5398
		rd	205	AS5399	AS5399
		rd	206	AS5400	AS5400
		rd	207	AS5401	AS5401
		rd	208	AS5402	AS5402
		rd	209	AS5403	AS5403
		rd	210	AS5404	AS5404
		rd	211	AS5405	AS5405
		rd	212	AS5406	AS5406
		rd	213	AS5407	AS5407
		rd	214	AS5408	AS5408
		rd	215	AS5409	AS5409
		rd	216	AS5410	AS5410
		rd	217	AS5411	AS5411
		rd	218	AS5412	AS5412
		rd	219	AS5413	AS5413
		rd	220	AS5414	AS5414
		rd	221	AS5415	AS5415
		rd	222	AS5416	AS5416
		rd	223	AS5417	AS5417
		rd	224	AS5418	AS5418
		rd	225	AS5419	AS5419
		rd	226	AS5420	AS5420
		rd	227	AS5421	AS5421
		rd	228	AS5422	AS5422
		rd	229	AS5423	AS5423
		rd	230	AS5424	AS5424
		rd	231	AS5425	AS5425
		rd	232	AS5426	AS5426
		rd	233	AS5427	AS5427
		rd	234	AS5428	AS5428
		rd	235	AS5429	AS5429
		rd	236	AS5430	AS5430
		rd	237	AS5431	AS5431
		rd	238	AS5432	AS5432
		rd	239	AS5433	AS5433
		rd	240	AS5434	AS5434
		rd	241	AS5435	AS5435
		rd	242	AS5436	AS5436
		rd	243	AS5437	AS5437
		rd	244	AS5438	AS5438
		rd	245	AS5439	AS5439
		rd	246	AS5440	AS5440
		rd	247	AS5441	AS5441
		rd	248	AS5442	AS5442
		rd	249	AS5443	AS5443
		rd	250	AS5444	AS5444
		rd	251	AS5445	AS5445
		rd	252	AS5446	AS5446
		rd	253	AS5447	AS5447
		rd	254	AS5448	AS5448
		rd	255	AS5449	AS5449
		rd	256	AS5450	AS5450
		rd	257	AS5451	AS5451
		rd	258	AS5452	AS5452
		rd	259	AS5453	AS5453
		rd	260	AS5454	AS5454
		rd	261	AS5455	AS5455
		rd	262	AS5456	AS5456
		rd	263	AS5457	AS5457
		rd	264	AS5458	AS5458
		rd	265	AS5459	AS5459
		rd	266	AS5460	AS5460
		rd	267	AS5461	AS5461
		rd	268	AS5462	AS5462
		rd	269	AS5463	AS5463
		rd	270	AS5464	AS5464

Figure 7.6 (cont)

Breakpoints		Size (bp)		SCG	Isolate
Start	End	MRU-WTR Cluster			
2436091	2436094	1	rd	1	RGB423
2436148	2436150	1	NC	2	ARS7886
2437845	2437846	2	rd	2	CC-5079
2437845	2437848	2	rd	2	NC250_09
2439162	2439163	1	rd	2	NC250_09
2441627	2441628	1	rd	2	NC250_09
2443495	2443501	1	rd	2	NC250_09
2443938	2443939	1	rd	2	NC250_09
2444514	2444515	1	rd	2	NC250_09
2444556	2444557	1	rd	2	NC250_09
2445785	2445786	1	rd	2	NC250_09
2447698	2447699	1	rd	2	NC250_09
2448521	2448522	1	rd	2	NC250_09
2448784	2448785	1	rd	2	NC250_09
2450882	2450883	1	rd	2	NC250_09
2450935	2450936	1	rd	2	NC250_09
2451271	2451272	1	rd	2	NC250_09
2451700	2451701	1	rd	2	NC250_09
2453753	2453754	1	rd	2	NC250_09
2453854	2453855	1	rd	2	NC250_09
2454126	2454127	2	rd	2	NC250_09
2454311	2454312	3	rd	2	NC250_09
2454318	2454319	3	rd	2	NC250_09
2454934	2454935	1	rd	2	NC250_09
2455261	2455262	2	rd	2	NC250_09
2455360	2455361	1	rd	2	NC250_09
2456224	2456225	1	rd	2	NC250_09
2456272	2456273	1	rd	2	NC250_09
2457903	2457904	1	rd	2	NC250_09
2458354	2458355	1	rd	2	NC250_09
2460428	2460429	2	rd	2	NC250_09
2460778	2460779	1	rd	2	NC250_09
2461547	2461548	1	rd	2	NC250_09
2462333	2462334	1	rd	2	NC250_09
2463708	2463709	1	rd	2	NC250_09
2464278	2464279	1	rd	2	NC250_09
2465338	2465339	1	rd	2	NC250_09
2465798	2465799	1	rd	2	NC250_09
2467798	2467799	1	rd	2	NC250_09
2469427	2469428	1	rd	2	NC250_09
2470058	2470059	1	rd	2	NC250_09
2470528	2470529	1	rd	2	NC250_09
2470558	2470559	1	rd	2	NC250_09
2470772	2470773	1	rd	2	NC250_09
2472310	2472311	1	rd	2	NC250_09
2474071	2474072	1	rd	2	NC250_09
2474174	2474175	1	rd	2	NC250_09
2475500	2475501	1	rd	2	NC250_09
2475900	2475901	1	rd	2	NC250_09
2478621	2478622	1	rd	2	NC250_09
2478899	2478900	1	rd	2	NC250_09
2481694	2481695	1	rd	2	NC250_09
2482898	2482899	1	rd	2	NC250_09
2484208	2484209	2	rd	2	NC250_09
2487411	2487412	1	rd	2	NC250_09
2487786	2487787	1	rd	2	NC250_09
2490144	2490145	2	rd	2	NC250_09
2490408	2490409	1	rd	2	NC250_09
2490712	2490713	1	rd	2	NC250_09
2490828	2490829	1	rd	2	NC250_09
2490903	2490904	1	rd	2	NC250_09
2491569	2491570	1	rd	2	NC250_09
2492256	2492257	1	rd	2	NC250_09
2492344	2492345	1	rd	2	NC250_09
2492432	2492433	1	rd	2	NC250_09
2492865	2492866	1	rd	2	NC250_09
2493172	2493173	1	rd	2	NC250_09
2497555	2497556	1	rd	2	NC250_09
2502183	2502184	1	rd	2	NC250_09
2502218	2502219	1	rd	2	NC250_09
2503407	2503408	1	rd	2	NC250_09
2503657	2503658	1	rd	2	NC250_09
2508708	2508709	1	rd	2	NC250_09
2509521	2509522	1	rd	2	NC250_09
2509921	2509922	1	rd	2	NC250_09
2510938	2510939	1	rd	2	NC250_09
2511071	2511072	1	rd	2	NC250_09
2511544	2511545	2	rd	2	NC250_09
2519432	2519433	1	rd	2	NC250_09
2519770	2519771	1	rd	2	NC250_09
2522481	2522482	1	rd	2	NC250_09
2523205	2523206	1	rd	2	NC250_09
2524563	2524564	1	rd	2	NC250_09
2525141	2525142	1	rd	2	NC250_09
2526388	2526389	1	rd	2	NC250_09
2530650	2530651	1	rd	2	NC250_09
2530874	2530875	1	rd	2	NC250_09
2530975	2530976	2	rd	2	NC250_09
2531486	2531487	1	rd	2	NC250_09
2534057	2534058	1	rd	2	NC250_09
2534811	2534812	1	rd	2	NC250_09
2534837	2534838	1	rd	2	NC250_09
2536625	2536626	1	rd	2	NC250_09
2536628	2536629	1	rd	2	NC250_09
2536688	2536689	1	rd	2	NC250_09
2538191	2538192	3	rd	2	NC250_09
2538377	2538378	1	rd	2	NC250_09
2540598	2540599	2	rd	2	NC250_09
2540647	2540648	1	rd	2	NC250_09
2541218	2541219	1	rd	2	NC250_09
2543583	2543584	1	rd	2	NC250_09
2545984	2545985	1	rd	2	NC250_09
2546038	2546039	1	rd	2	NC250_09
2546508	2546509	1	rd	2	NC250_09
2547172	2547173	1	rd	2	NC250_09
2547253	2547254	1	rd	2	NC250_09
2547691	2547692	1	rd	2	NC250_09
2550103	2550104	1	rd	2	NC250_09
2552984	2552985	1	rd	2	NC250_09
2553933	2553934	1	rd	2	NC250_09
2555505	2555506	1	rd	2	NC250_09
2555866	2555867	1	rd	2	NC250_09
2557214	2557215	1	rd	2	NC250_09
2557978	2557979	1	rd	2	NC250_09
2558572	2558573	1	rd	2	NC250_09
2560134	2560135	2	rd	2	NC250_09
2560593	2560594	1	rd	2	NC250_09
2560643	2560644	1	rd	2	NC250_09
2561703	2561704	2	rd	2	NC250_09
2562478	2562479	1	rd	2	NC250_09
2562534	2562535	1	rd	2	NC250_09
2562999	2562999	1	rd	2	NC250_09
2564368	2564369	1	rd	2	NC250_09
2565302	2565303	1	rd	2	NC250_09
2569865	2569866	2	rd	2	NC250_09
2569918	2569919	1	rd	2	NC250_09
2571395	2571396	1	rd	2	NC250_09
2571446	2571447	1	rd	2	NC250_09
2572268	2572269	1	rd	2	NC250_09
2572318	2572319	1	rd	2	NC250_09
2575508	2575509	1	rd	2	NC250_09
2576435	2576436	1	rd	2	NC250_09
2576634	2576635	1	rd	2	NC250_09
2576754	2576755	3	rd	2	NC250_09
2577208	2577209	1	rd	2	NC250_09
2577371	2577372	4	rd	2	NC250_09
2578471	2578472	1	rd	2	NC250_09
2580411	2580412	2	rd	2	NC250_09
2581408	2581409	1	rd	2	NC250_09
2581457	2581458	1	rd	2	NC250_09
2582152	2582153	2	rd	2	NC250_09
2583188	2583189	1	rd	2	NC250_09
2585437	2585438	1	rd	2	NC250_09
2588332	2588333	1	rd	2	NC250_09
2589511	2589512	1	rd	2	NC250_09
2591377	2591378	1	rd	2	NC250_09
2592688	2592689	1	rd	2	NC250_09
2594358	2594359	1	rd	2	NC250_09
2594551	2594552	1	rd	2	NC250_09
2597768	2597769	1	rd	2	NC250_09
2598993	2598994	1	rd	2	NC250_09
2599608	2599609	2	rd	2	NC250_09
Total					60

Figure 7.6 (cont)

Breakpoints		Size (bp)	SCG	MRU-WTR Cluster	Isolate
Start	End				
					1
2600493	2600494	1		rd	IGTB423
2601625	2601630	1		NC	ARS7896
2603383	2603388	1		rd	CC25079
2604158	2604157	9		rd	NC2750_09
2605419	2605416	1		rd	NC2750_09
2605797	2605798	1		rd	NC2750_09
2607138	2607133	1		rd	NC2750_09
2608845	2608855	1		rd	NC2750_09
2608950	2608957	1		rd	NC2750_09
2610353	2610358	1		rd	NC2750_09
2615475	2615480	1		rd	NC2750_09
2617248	2617250	3		rd	NC2750_09
2617250	2617250	1		rd	NC2750_09
2617310	2617311	1		rd	NC2750_09
2617637	2617638	1		rd	NC2750_09
2618363	2618364	1		rd	NC2750_09
2618561	2618564	1		rd	NC2750_09
2619001	2619003	1		rd	NC2750_09
2620914	2620917	1		rd	NC2750_09
2621358	2621359	1		rd	NC2750_09
2626911	2626912	1		rd	NC2750_09
2630245	2630246	1		rd	NC2750_09
2631009	2631010	5		rd	NC2750_09
2632141	2632144	1		rd	NC2750_09
2633060	2633063	1		rd	NC2750_09
2633972	2633977	1		rd	NC2750_09
2634999	2635000	4		rd	NC2750_09
2635545	2635546	30		rd	NC2750_09
2635576	2635577	45		rd	NC2750_09
2636925	2636930	27		rd	NC2750_09
2638081	2638084	1		rd	NC2750_09
2639461	2639462	1		rd	NC2750_09
2645285	2645288	2		rd	NC2750_09
2645542	2645543	1		rd	NC2750_09
2648845	2648846	2		rd	NC2750_09
2648998	2648999	1		rd	NC2750_09
2650459	2650460	1		rd	NC2750_09
2651654	2651655	2		rd	NC2750_09
2651966	2651970	1		rd	NC2750_09
2652148	2652147	1		rd	NC2750_09
2654910	2654911	1		rd	NC2750_09
2656794	2656795	1		rd	NC2750_09
2657070	2657077	1		rd	NC2750_09
2658226	2658227	1		rd	NC2750_09
2659681	2659688	1		rd	NC2750_09
2663924	2663927	2		rd	NC2750_09
2667998	2667999	1		rd	NC2750_09
2668530	2668531	1		rd	NC2750_09
2671471	2671474	1		rd	NC2750_09
2672166	2672170	1		rd	NC2750_09
2672989	2672990	2		rd	NC2750_09
2674831	2674838	1		rd	NC2750_09
2676551	2676552	1		rd	NC2750_09
2677786	2677787	1		rd	NC2750_09
2680044	2680045	1		rd	NC2750_09
2681446	2681446	1		rd	NC2750_09
2682911	2682914	1		rd	NC2750_09
2683952	2683953	1		rd	NC2750_09
2685981	2685988	1		rd	NC2750_09
2686051	2686052	1		rd	NC2750_09
2686370	2686377	1		rd	NC2750_09
2686421	2686422	1		rd	NC2750_09
2686510	2686511	1		rd	NC2750_09
2686658	2686659	1		rd	NC2750_09
2687091	2687092	1		rd	NC2750_09
2687526	2687527	2		rd	NC2750_09
2688467	2688468	1		rd	NC2750_09
2688869	2688870	1		rd	NC2750_09
2690434	2690435	1		rd	NC2750_09
2692528	2692530	1		rd	NC2750_09
2692710	2692711	1		rd	NC2750_09
2692759	2692760	1		rd	NC2750_09
2692824	2692825	1		rd	NC2750_09
2693952	2693953	9		rd	NC2750_09
2694434	2694435	2		rd	NC2750_09
2694480	2694482	2		rd	NC2750_09
2693999	2694000	1		rd	NC2750_09
2694151	2694158	2		rd	NC2750_09
2696232	2696233	1		rd	NC2750_09
2703902	2703903	1		rd	NC2750_09
2704884	2704885	21		rd	NC2750_09
2704885	2704886	21		rd	NC2750_09
2706873	2706874	2		rd	NC2750_09
2708105	2708106	1		rd	NC2750_09
2708501	2708508	1		rd	NC2750_09
2709670	2709671	2		rd	NC2750_09
2710998	2710999	1		rd	NC2750_09
2711774	2711775	1		rd	NC2750_09
2711298	2711299	2		rd	NC2750_09
2713076	2713077	3		rd	NC2750_09
2713365	2713366	1		rd	NC2750_09
2713841	2713842	1		rd	NC2750_09
2714298	2714297	2		rd	NC2750_09
2714871	2714872	3		rd	NC2750_09
2715249	2715246	1		rd	NC2750_09
2715577	2715578	4		rd	NC2750_09
2717291	2717292	1		rd	NC2750_09
2720634	2720635	1		rd	NC2750_09
2723185	2723188	2		rd	NC2750_09
2728442	2728443	2		rd	NC2750_09
2728544	2728545	1		rd	NC2750_09
2728686	2728687	1		rd	NC2750_09
2728938	2728939	1		rd	NC2750_09
2731083	2731088	1		rd	NC2750_09
2732166	2732167	1		rd	NC2750_09
2732486	2732485	1		rd	NC2750_09
2733991	2733994	1		rd	NC2750_09
2736489	2736490	1		rd	NC2750_09
2740178	2740175	1		rd	NC2750_09
2741095	2741096	1		rd	NC2750_09
2741095	2741100	1		rd	NC2750_09
2743410	2743412	3		rd	NC2750_09
2748192	2748193	1		rd	NC2750_09
2748315	2748320	1		rd	NC2750_09
2748722	2748728	1		rd	NC2750_09
2748999	2748999	1		rd	NC2750_09
2750311	2750314	1		rd	NC2750_09
2750635	2750636	1		rd	NC2750_09
2750666	2750667	1		rd	NC2750_09
2751558	2751559	1		rd	NC2750_09
2752261	2752262	3		rd	NC2750_09
2753196	2753199	2		rd	NC2750_09
2753661	2753668	1		rd	NC2750_09
2755000	2755003	1		rd	NC2750_09
2755032	2755033	2		rd	NC2750_09
2755301	2755308	1		rd	NC2750_09
2756422	2756423	1		rd	NC2750_09
2756870	2756877	1		rd	NC2750_09
2757708	2757705	1		rd	NC2750_09
2758354	2758357	1		rd	NC2750_09
2758876	2758877	1		rd	NC2750_09
2762263	2762262	3		rd	NC2750_09
2762688	2762685	1		rd	NC2750_09
2764578	2764575	1		rd	NC2750_09
2764882	2764883	1		rd	NC2750_09
2767395	2767396	2		rd	NC2750_09
2769471	2769478	1		rd	NC2750_09
2769525	2769536	1		rd	NC2750_09
2770052	2770053	1		rd	NC2750_09
2770388	2770385	1		rd	NC2750_09
2771871	2771872	1		rd	NC2750_09
2772222	2772223	1		rd	NC2750_09
2773164	2773165	1		rd	NC2750_09
2773621	2773622	1		rd	NC2750_09
2774154	2774155	1		rd	NC2750_09
2774771	2774772	1		rd	NC2750_09
2777433	2777438	1		rd	NC2750_09
2777748	2777747	2		rd	NC2750_09
2777864	2777865	1		rd	NC2750_09
2778910	2778911	1		rd	NC2750_09
2779400	2779403	1		rd	NC2750_09
2782874	2782875	1		rd	NC2750_09
Total					18





Figure 7.6 (cont)

Breakpoints		Size (bp)	SCG	Isolate
Start	End		MRU-WTR Cluster	
3119575	3119576	1	rd	IGCB423
3119595	3119596	1	NC	ARS7886
3119608	3119609	1	NC	CC-5079
3119705	3119706	1	rd	NC250_09
3120000	3120001	20	NC	NC250_09
3120061	3120062	1	rd	NC
3120151	3120152	1	rd	NC
3120523	3120524	13	rd	NC
3122052	3122053	1	rd	NC
3122266	3122267	79	rd	NC
3122455	3122456	1	rd	NC
3122493	3122494	1	rd	NC
3122810	3122811	12	rd	NC
3122954	3122955	15	rd	NC
3122970	3122971	1	rd	NC
3123048	3123049	1	rd	NC
3123194	3123195	1	rd	NC
3123195	3123196	2	rd	NC
3123388	3123389	4	rd	NC
3127711	3127712	1	rd	NC
3131461	3131462	9	rd	NC
3131478	3131479	9	rd	NC
3134995	3134996	1	rd	NC
3136971	3136972	1	rd	NC
3137715	3137716	1	rd	NC
3137942	3137943	1	rd	NC
3138565	3138566	2	rd	NC
3141444	3141445	1	rd	NC
3141508	3141509	1	rd	NC
3141912	3141913	1	rd	NC
3143121	3143122	1	rd	NC
3145744	3145745	3	rd	NC
3145948	3145949	1	rd	NC
3146278	3146279	2	rd	NC
3146445	3146446	1	rd	NC
3146721	3146722	1	rd	NC
3147955	3147956	1	rd	NC
3147998	3147999	2	rd	NC
3150225	3150226	1	rd	NC
3151998	3151999	1	rd	NC
3152033	3152034	1	rd	NC
3156166	3156167	1	rd	NC
3156201	3156202	1	rd	NC
3156846	3156847	2	rd	NC
3158316	3158317	1	rd	NC
3158485	3158486	1	rd	NC
3159322	3159323	2	rd	NC
3160331	3160332	1	rd	NC
3163008	3163009	1	rd	NC
3163708	3163709	25	rd	NC
3163815	3163816	2	rd	NC
3163861	3163862	24	rd	NC
3164571	3164572	1	rd	NC
3167064	3167065	1	rd	NC
3168261	3168262	1	rd	NC
3171991	3171992	2	rd	NC
3173474	3173475	1	rd	NC
3173608	3173609	1	rd	NC
3173631	3173632	1	rd	NC
3177528	3177529	1	rd	NC
3177585	3177586	1	rd	NC
3179195	3179196	1	rd	NC
3179724	3179725	1	rd	NC
3180291	3180292	2	rd	NC
3188188	3188189	1	rd	NC
3192708	3192709	3	rd	NC
3193425	3193426	2	rd	NC
3194243	3194244	1	rd	NC
3194660	3194661	1	rd	NC
3194714	3194715	2	rd	NC
3197063	3197064	1	rd	NC
3197845	3197846	1	rd	NC
3200096	3200097	1	rd	NC
3200930	3200931	1	rd	NC
3200936	3200937	1	rd	NC
3203505	3203506	1	rd	NC
3204895	3204896	3	rd	NC
3207711	3207712	1	rd	NC
3209105	3209106	2	rd	NC
3209870	3209871	1	rd	NC
3209955	3209956	1	rd	NC
3209995	3209996	1	rd	NC
3215478	3215479	1	rd	NC
3217715	3217716	1	rd	NC
3218166	3218167	1	rd	NC
3219451	3219452	1	rd	NC
3220693	3220694	1	rd	NC
3220778	3220779	1	rd	NC
3221516	3221517	1	rd	NC
3224423	3224424	1	rd	NC
3225411	3225412	1	rd	NC
3225982	3225983	1	rd	NC
3227478	3227479	1	rd	NC
3227915	3227916	1	rd	NC
3228996	3228997	1	rd	NC
3229595	3229596	1	rd	NC
3230400	3230401	7	rd	NC
3230721	3230722	1	rd	NC
3230985	3230986	1	rd	NC
3232125	3232126	2	rd	NC
3232188	3232189	1	rd	NC
3232995	3232996	1	rd	NC
3233500	3233501	1	rd	NC
3234073	3234074	1	rd	NC
3236793	3236794	1	rd	NC
3240073	3240074	1	rd	NC
3240985	3240986	2	rd	NC
3243065	3243066	1	rd	NC
3243828	3243829	1	rd	NC
3248308	3248309	1	rd	NC
3248430	3248431	3	rd	NC
3248472	3248473	1	rd	NC
3248995	3248996	1	rd	NC
3250004	3250005	1	rd	NC
3250726	3250727	2	rd	NC
3252246	3252247	1	rd	NC
3253225	3253226	2	rd	NC
3253681	3253682	1	rd	NC
3255614	3255615	1	rd	NC
3255995	3255996	1	rd	NC
3256056	3256057	1	rd	NC
3257400	3257401	2	rd	NC
3261571	3261572	2	rd	NC
3261725	3261726	1	rd	NC
3262355	3262356	1	rd	NC
3263296	3263297	1	rd	NC
3263396	3263397	1	rd	NC
3263420	3263421	2	rd	NC
3263971	3263972	1	rd	NC
3263998	3263999	1	rd	NC
3265444	3265445	1	rd	NC
3266063	3266064	1	rd	NC
3270155	3270156	2	rd	NC
3270615	3270616	1	rd	NC
3273855	3273856	1	rd	NC
3275801	3275802	1	rd	NC
3280810	3280811	1	rd	NC
3281571	3281572	1	rd	NC
3283241	3283242	2	rd	NC
3284065	3284066	4	rd	NC
3284605	3284606	1	rd	NC
3284655	3284656	1	rd	NC
3286463	3286464	1	rd	NC
3287995	3287996	1	rd	NC
3289081	3289082	1	rd	NC
3290671	3290672	1	rd	NC
3290731	3290732	1	rd	NC
3292761	3292762	1	rd	NC
3293371	3293372	7	rd	NC
3297611	3297612	1	rd	NC
3297664	3297665	2	rd	NC
3301604	3301605	1	rd	NC
		Total		

Figure 7.6 (cont)

Breakpoints		Size (bp)		SCG	Isolate
Start	End	MRU-WTR Cluster			
			1	1	1
			2	2	2
			3	3	3
			4	4	4
			5	5	5
			6	6	6
			7	7	7
			8	8	8
			9	9	9
			10	10	10
			11	11	11
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			294	294	294
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Figure 7.6 (cont)

Breakpoints		Size (bp)		SCG	MIRU-VNR Cluster	Isolate
Start	End					
3488135	3488136	1		rd	1	IGB423
3488860	3488861	1		NC	2	ARS7886
3489386	3489387	1		rd	2	CC25079
3489733	3489734	1		rd	2	NC2502_09
3493339	3493340	1		rd	2	NC2502_09
3495784	3495785	1		rd	2	NC2502_09
3497192	3497193	1		rd	2	NC2502_09
3497741	3497742	1		rd	2	NC2502_09
3499592	3499593	1		rd	2	NC2502_09
3500353	3500354	1		rd	2	NC2502_09
3500388	3500389	1		rd	2	NC2502_09
3501323	3501324	1		rd	2	NC2502_09
3501721	3501722	1		rd	2	NC2502_09
3502261	3502262	1		rd	2	NC2502_09
3507951	3507952	1		rd	2	NC2502_09
3509585	3509586	1		rd	2	NC2502_09
3509638	3509639	1		rd	2	NC2502_09
3510226	3510227	1		rd	2	NC2502_09
3512083	3512084	1		rd	2	NC2502_09
3514911	3514912	1		rd	2	NC2502_09
3515899	3515900	1		rd	2	NC2502_09
3516109	3516110	1		rd	2	NC2502_09
3516386	3516387	1		rd	2	NC2502_09
3516720	3516721	1		rd	2	NC2502_09
3516961	3516962	1		rd	2	NC2502_09
3518409	3518410	1		rd	2	NC2502_09
3519294	3519295	1		rd	2	NC2502_09
3519356	3519357	1		rd	2	NC2502_09
3521477	3521478	1		rd	2	NC2502_09
3524393	3524394	1		rd	2	NC2502_09
3524508	3524509	1		rd	2	NC2502_09
3524522	3524523	1		rd	2	NC2502_09
3528411	3528412	1		rd	2	NC2502_09
3528608	3528609	1		rd	2	NC2502_09
3532031	3532032	1		rd	2	NC2502_09
3533627	3533628	1		rd	2	NC2502_09
3536198	3536199	1		rd	2	NC2502_09
3537000	3537001	1		rd	2	NC2502_09
3540824	3540825	1		rd	2	NC2502_09
3541964	3541965	1		rd	2	NC2502_09
3543338	3543339	1		rd	2	NC2502_09
3544001	3544002	1		rd	2	NC2502_09
3544122	3544123	1		rd	2	NC2502_09
3546910	3546911	1		rd	2	NC2502_09
3546950	3546951	1		rd	2	NC2502_09
3548750	3548751	1		rd	2	NC2502_09
3549744	3549745	1		rd	2	NC2502_09
3549999	3549999	1		rd	2	NC2502_09
3550288	3550289	1		rd	2	NC2502_09
3550331	3550332	1		rd	2	NC2502_09
3554364	3554365	1		rd	2	NC2502_09
3555184	3555185	1		rd	2	NC2502_09
3555508	3555509	1		rd	2	NC2502_09
3556382	3556383	1		rd	2	NC2502_09
3556800	3556801	1		rd	2	NC2502_09
3556894	3556895	1		rd	2	NC2502_09
3557135	3557136	1		rd	2	NC2502_09
3557818	3557819	1		rd	2	NC2502_09
3557873	3557874	1		rd	2	NC2502_09
3561881	3561882	1		rd	2	NC2502_09
3563602	3563603	1		rd	2	NC2502_09
3565155	3565156	1		rd	2	NC2502_09
3566500	3566501	1		rd	2	NC2502_09
3572110	3572111	1		rd	2	NC2502_09
3572640	3572641	1		rd	2	NC2502_09
3572978	3572979	1		rd	2	NC2502_09
3573505	3573506	1		rd	2	NC2502_09
3574553	3574554	1		rd	2	NC2502_09
3575700	3575701	1		rd	2	NC2502_09
3576477	3576478	1		rd	2	NC2502_09
3576534	3576535	1		rd	2	NC2502_09
3578183	3578184	1		rd	2	NC2502_09
3578858	3578859	1		rd	2	NC2502_09
3581200	3581201	1		rd	2	NC2502_09
3581877	3581878	1		rd	2	NC2502_09
3582890	3582891	1		rd	2	NC2502_09
3583558	3583559	1		rd	2	NC2502_09
3585572	3585573	1		rd	2	NC2502_09
3588452	3588453	1		rd	2	NC2502_09
3589215	3589216	1		rd	2	NC2502_09
3590686	3590687	1		rd	2	NC2502_09
3590745	3590746	1		rd	2	NC2502_09
3590846	3590847	1		rd	2	NC2502_09
3593611	3593612	1		rd	2	NC2502_09
3594461	3594462	1		rd	2	NC2502_09
3595483	3595484	1		rd	2	NC2502_09
3598510	3598511	1		rd	2	NC2502_09
3598998	3598999	1		rd	2	NC2502_09
3600198	3600199	1		rd	2	NC2502_09
3600948	3600949	1		rd	2	NC2502_09
3601288	3601289	1		rd	2	NC2502_09
3602344	3602345	1		rd	2	NC2502_09
3602754	3602755	1		rd	2	NC2502_09
3604765	3604766	1		rd	2	NC2502_09
3605225	3605226	1		rd	2	NC2502_09
3606199	3606200	1		rd	2	NC2502_09
3608178	3608179	1		rd	2	NC2502_09
3609793	3609794	1		rd	2	NC2502_09
3610033	3610034	1		rd	2	NC2502_09
3610088	3610089	1		rd	2	NC2502_09
3610393	3610394	1		rd	2	NC2502_09
3610734	3610735	1		rd	2	NC2502_09
3612384	3612385	1		rd	2	NC2502_09
3612932	3612933	1		rd	2	NC2502_09
3612978	3612979	1		rd	2	NC2502_09
3615933	3615934	1		rd	2	NC2502_09
3615986	3615987	1		rd	2	NC2502_09
3616130	3616131	1		rd	2	NC2502_09
3617077	3617078	1		rd	2	NC2502_09
3618761	3618762	1		rd	2	NC2502_09
3620384	3620385	1		rd	2	NC2502_09
3623941	3623942	1		rd	2	NC2502_09
3624055	3624056	1		rd	2	NC2502_09
3625347	3625348	1		rd	2	NC2502_09
3626228	3626229	1		rd	2	NC2502_09
3626610	3626611	1		rd	2	NC2502_09
3627400	3627401	1		rd	2	NC2502_09
3627625	3627626	1		rd	2	NC2502_09
3627676	3627677	1		rd	2	NC2502_09
3629232	3629233	1		rd	2	NC2502_09
3631226	3631227	1		rd	2	NC2502_09
3633186	3633187	1		rd	2	NC2502_09
3636105	3636106	1		rd	2	NC2502_09
3636178	3636179	1		rd	2	NC2502_09
3637278	3637279	1		rd	2	NC2502_09
3639810	3639811	1		rd	2	NC2502_09
3640381	3640382	1		rd	2	NC2502_09
3640805	3640806	1		rd	2	NC2502_09
3640953	3640954	1		rd	2	NC2502_09
3641394	3641395	1		rd	2	NC2502_09
3642309	3642310	1		rd	2	NC2502_09
3643333	3643334	1		rd	2	NC2502_09
3643509	3643510	1		rd	2	NC2502_09
3644778	3644779	1		rd	2	NC2502_09
3646698	3646699	1		rd	2	NC2502_09
3650283	3650284	1		rd	2	NC2502_09
3650433	3650434	1		rd	2	NC2502_09
3650961	3650962	1		rd	2	NC2502_09
3650997	3650998	1		rd	2	NC2502_09
3651188	3651189	1		rd	2	NC2502_09
3651523	3651524	1		rd	2	NC2502_09
3651577	3651578	1		rd	2	NC2502_09
3652410	3652411	1		rd	2	NC2502_09
3655063	3655064	1		rd	2	NC2502_09
3657813	3657814	1		rd	2	NC2502_09
3657986	3657987	1		rd	2	NC2502_09
3658388	3658389	1		rd	2	NC2502_09
3659311	3659312	1		rd	2	NC2502_09
3660755	3660756	1		rd	2	NC2502_09
3661842	3661843	1		rd	2	NC2502_09
3665253	3665254	1		rd	2	NC2502_09
3665380	3665381	1		rd	2	NC2502_09
Total						18

Figure 7.6 (cont)

Breakpoints		Size (bp)		MRU-WTR Cluster	SCG	Isolate
Start	End					
3665752	3665758	5		rd	1	IGTB423
3665763	3665762	1		NC	2	ARS7886
3665813	3665814	2		rd	2	CC35079
3670073	3670074	2		rd	2	NC250_09
3671193	3671194	1		NC	2	NC250_09
3672164	3672165	1		rd	2	HM7295_08
3672221	3672224	1		rd	2	HM769_11
3673658	3673655	35		rd	2	R1307
3674208	3674205	1		rd	2	K122
3675425	3675426	1		rd	2	ARS9427
3675801	3675808	1		rd	2	NC250_09
3678126	3678127	1		rd	2	NC250_09
3678181	3678182	1		rd	2	NC250_09
3678211	3678218	1		rd	2	NC250_09
3678900	3678907	1		rd	2	NC250_09
3679976	3679977	1		rd	2	NC250_09
3684192	3684193	2		rd	2	NC250_09
3684577	3684578	1		rd	2	NC250_09
3684992	3684993	1		rd	2	NC250_09
3685491	3685492	1		rd	2	NC250_09
3685676	3685677	1		rd	2	NC250_09
3686605	3686610	1		rd	2	NC250_09
3687901	3687904	1		rd	2	NC250_09
3690218	3690219	2		rd	2	NC250_09
3691005	3691008	58		rd	2	NC250_09
3691154	3691155	3		rd	2	NC250_09
3691335	3691336	1		rd	2	NC250_09
3692478	3692479	2		rd	2	NC250_09
3692532	3692533	1		rd	2	NC250_09
3695245	3695246	1		rd	2	NC250_09
3695886	3695887	1		rd	2	NC250_09
3696373	3696374	1		rd	2	NC250_09
3696425	3696426	1		rd	2	NC250_09
3696598	3696599	1		rd	2	NC250_09
3696598	3696599	1		rd	2	NC250_09
3696810	3696812	1		rd	2	NC250_09
3700105	3700106	1		rd	2	NC250_09
3700212	3700213	1		rd	2	NC250_09
3704305	3704306	1		rd	2	NC250_09
3705142	3705143	1		rd	2	NC250_09
3705741	3705744	1		rd	2	NC250_09
3707054	3707055	2		rd	2	NC250_09
3708196	3708198	1		rd	2	NC250_09
3709254	3709255	1		rd	2	NC250_09
3710001	3710004	4		rd	2	NC250_09
3712648	3712647	1		rd	2	NC250_09
3712761	3712762	1		rd	2	NC250_09
3713908	3713909	1		rd	2	NC250_09
3714877	3714878	1		rd	2	NC250_09
3715622	3715623	1		rd	2	NC250_09
3719678	3719679	1		rd	2	NC250_09
3720954	3720955	1		rd	2	NC250_09
3722821	3722822	1		rd	2	NC250_09
3723581	3723582	1		rd	2	NC250_09
3723901	3723902	1		rd	2	NC250_09
3723988	3723990	1		rd	2	NC250_09
3725601	3725602	1		rd	2	NC250_09
3725606	3725607	1		rd	2	NC250_09
3725666	3725665	1		rd	2	NC250_09
3726914	3726915	1		rd	2	NC250_09
3727384	3727385	1		rd	2	NC250_09
3729315	3729316	1		rd	2	NC250_09
3729665	3729666	36		rd	2	NC250_09
3731118	3731119	1		rd	2	NC250_09
3731501	3731502	1		rd	2	NC250_09
3731223	3731224	1		rd	2	NC250_09
3732378	3732379	1		rd	2	NC250_09
3733488	3733487	1		rd	2	NC250_09
3733541	3733544	1		rd	2	NC250_09
3735332	3735333	1		rd	2	NC250_09
3735850	3735853	3		rd	2	NC250_09
3736008	3736009	54		rd	2	NC250_09
3737234	3737235	1		rd	2	NC250_09
3737331	3737333	1		rd	2	NC250_09
3737743	3737744	1		rd	2	NC250_09
3737971	3737972	1		rd	2	NC250_09
3738111	3738114	1		rd	2	NC250_09
3738430	3738437	1		rd	2	NC250_09
3738516	3738517	18		rd	2	NC250_09
3738728	3738729	8		rd	2	NC250_09
3739214	3739215	1		rd	2	NC250_09
3739375	3739376	1		rd	2	NC250_09
3739391	3739392	1		rd	2	NC250_09
3739628	3739629	1		rd	2	NC250_09
3740765	3740766	13		rd	2	NC250_09
3740853	3740855	1		rd	2	NC250_09
3740931	3740933	1		rd	2	NC250_09
3741285	3741286	18		rd	2	NC250_09
3741355	3741360	1		rd	2	NC250_09
3741905	3741910	1		rd	2	NC250_09
3742921	3742924	1		rd	2	NC250_09
3744025	3744030	1		rd	2	NC250_09
3744200	3744203	1		rd	2	NC250_09
3746661	3746663	1		rd	2	NC250_09
3751266	3751267	1		rd	2	NC250_09
3752471	3752472	1		rd	2	NC250_09
3752935	3752940	1		rd	2	NC250_09
3755561	3755568	1		rd	2	NC250_09
3758444	3758445	1		rd	2	NC250_09
3759812	3759813	1		rd	2	NC250_09
3761411	3761414	2		rd	2	NC250_09
3761470	3761477	1		rd	2	NC250_09
3763245	3763246	1		rd	2	NC250_09
3764301	3764308	1		rd	2	NC250_09
3765665	3765668	1		rd	2	NC250_09
3765881	3765882	1		rd	2	NC250_09
3771260	3771263	2		rd	2	NC250_09
3771315	3771316	1		rd	2	NC250_09
3771365	3771366	1		rd	2	NC250_09
3771831	3771833	1		rd	2	NC250_09
3772150	3772153	1		rd	2	NC250_09
3773171	3773178	2		rd	2	NC250_09
3775151	3775153	1		rd	2	NC250_09
3777275	3777276	1		rd	2	NC250_09
3777331	3777338	1		rd	2	NC250_09
3779038	3779039	1		rd	2	NC250_09
3779671	3779672	9		rd	2	NC250_09
3779674	3779675	1		rd	2	NC250_09
3779752	3779753	1		rd	2	NC250_09
3779861	3779864	1		rd	2	NC250_09
3780608	3780609	1		rd	2	NC250_09
3782036	3782037	2		rd	2	NC250_09
3782111	3782118	1		rd	2	NC250_09
3782585	3782590	1		rd	2	NC250_09
3782851	3782853	1		rd	2	NC250_09
3782905	3782910	1		rd	2	NC250_09
3783336	3783333	2		rd	2	NC250_09
3783643	3783644	5		rd	2	NC250_09
3784995	3785000	1		rd	2	NC250_09
3786071	3786073	1		rd	2	NC250_09
3794805	3794806	1		rd	2	NC250_09
3794861	3794868	2		rd	2	NC250_09
3795125	3795126	1		rd	2	NC250_09
3795485	3795486	1		rd	2	NC250_09
3801180	3801181	1		rd	2	NC250_09
3801236	3801233	1		rd	2	NC250_09
3801291	3801293	1		rd	2	NC250_09
3801581	3801582	1		rd	2	NC250_09
3801635	3801636	1		rd	2	NC250_09
3802361	3802364	2		rd	2	NC250_09
3802446	3802447	1		rd	2	NC250_09
3802704	3802705	1		rd	2	NC250_09
3802785	3802790	4		rd	2	NC250_09
3802866	3802869	2		rd	2	NC250_09
3803000	3803001	5		rd	2	NC250_09
3803132	3803133	1		rd	2	NC250_09
3805571	3805576	1		rd	2	NC250_09
3807866	3807869	1		rd	2	NC250_09
3808218	3808219	1		rd	2	NC250_09
3808243	3808244	1		rd	2	NC250_09
3808299	3808298	1		rd	2	NC250_09
Total						40

Breakpoints		Size (bp)		MRU-WTR Cluster	SCG	Isolate
Start	End					
3808522	3808523	1		rd	1	IGTB423
3808950	3808951	1		NC	2	ARS7886
3809593	3809593	1		rd	2	CC35079
3809883	3809883	1		rd	2	NC252_09
3811107	3811108	1		rd	2	NC252_09
3811121	3811124	3		rd	2	NC252_09
3811495	3811500	5		rd	2	NC252_09
3811834	3811835	1		rd	2	NC252_09
3814053	3814052	1		rd	2	NC252_09
3815393	3815394	1		rd	2	NC252_09
3816094	3816095	1		rd	2	NC252_09
3816261	3816268	7		rd	2	NC252_09
3820281	3820285	4		rd	2	NC252_09
3820475	3820476	1		rd	2	NC252_09
3820501	3820504	3		rd	2	NC252_09
3824745	3824746	1		rd	2	NC252_09
3824774	3824775	1		rd	2	NC252_09
3827958	3827959	1		rd	2	NC252_09
3828355	3828360	5		rd	2	NC252_09
3831999	3832000	1		rd	2	NC252_09
3835277	3835278	1		rd	2	NC252_09
3835329	3835330	1		rd	2	NC252_09
3836085	3836086	1		rd	2	NC252_09
3836565	3836566	1		rd	2	NC252_09
3840485	3840486	1		rd	2	NC252_09
3841181	3841188	7		rd	2	NC252_09
3841197	3841198	1		rd	2	NC252_09
3841238	3841240	2		rd	2	NC252_09
3841466	3841467	1		rd	2	NC252_09
3842494	3842495	1		rd	2	NC252_09
3842760	3842768	8		rd	2	NC252_09
3842888	3842890	2		rd	2	NC252_09
3844294	3844295	1		rd	2	NC252_09
3844345	3844346	1		rd	2	NC252_09
3844748	3844747	1		rd	2	NC252_09
3845104	3845105	1		rd	2	NC252_09
3848272	3848273	1		rd	2	NC252_09
3851511	3851512	1		rd	2	NC252_09
3851893	3851893	1		rd	2	NC252_09
3851995	3852000	5		rd	2	NC252_09
3852070	3852077	7		rd	2	NC252_09
3853554	3853555	1		rd	2	NC252_09
3853708	3853709	1		rd	2	NC252_09
3854330	3854331	1		rd	2	NC252_09
3854468	3854469	1		rd	2	NC252_09
3855063	3855064	1		rd	2	NC252_09
3856188	3856189	1		rd	2	NC252_09
3857541	3857544	3		rd	2	NC252_09
3857596	3857599	3		rd	2	NC252_09
3857783	3857784	1		rd	2	NC252_09
3857788	3857789	1		rd	2	NC252_09
3859222	3859223	1		rd	2	NC252_09
3860448	3860447	1		rd	2	NC252_09
3860468	3860469	1		rd	2	NC252_09
3862191	3862192	1		rd	2	NC252_09
3862410	3862411	1		rd	2	NC252_09
3862433	3862434	1		rd	2	NC252_09
3863912	3863913	1		rd	2	NC252_09
3865325	3865326	1		rd	2	NC252_09
3865405	3865406	1		rd	2	NC252_09
3866398	3866399	1		rd	2	NC252_09
3866684	3866685	1		rd	2	NC252_09
3869800	3869801	1		rd	2	NC252_09
3869993	3869994	1		rd	2	NC252_09
3870624	3870626	2		rd	2	NC252_09
3871811	3871812	1		rd	2	NC252_09
3872166	3872167	1		rd	2	NC252_09
3875873	3875874	1		rd	2	NC252_09
3876428	3876429	1		rd	2	NC252_09
3877950	3877951	1		rd	2	NC252_09
3878555	3878556	1		rd	2	NC252_09
3878621	3878622	1		rd	2	NC252_09
3879395	3879396	1		rd	2	NC252_09
3879984	3879985	1		rd	2	NC252_09
3880238	3880240	2		rd	2	NC252_09
3880581	3880590	9		rd	2	NC252_09
3883038	3883039	1		rd	2	NC252_09
3884238	3884239	1		rd	2	NC252_09
3886511	3886514	3		rd	2	NC252_09
3887196	3887197	1		rd	2	NC252_09
3887943	3887948	5		rd	2	NC252_09
3888207	3888208	1		rd	2	NC252_09
3888316	3888317	1		rd	2	NC252_09
3889005	3889006	1		rd	2	NC252_09
3892643	3892644	1		rd	2	NC252_09
3892238	3892239	1		rd	2	NC252_09
3892584	3892585	1		rd	2	NC252_09
3892885	3892886	1		rd	2	NC252_09
3898827	3898828	1		rd	2	NC252_09
3902318	3902319	1		rd	2	NC252_09
3902844	3902845	1		rd	2	NC252_09
3902995	3902996	1		rd	2	NC252_09
3906185	3906186	1		rd	2	NC252_09
3906464	3906465	1		rd	2	NC252_09
3906653	3906654	1		rd	2	NC252_09
3906995	3907000	5		rd	2	NC252_09
3907998	3907999	1		rd	2	NC252_09
3909604	3909605	1		rd	2	NC252_09
3909714	3909715	1		rd	2	NC252_09
3911993	3911998	5		rd	2	NC252_09
3912194	3912195	1		rd	2	NC252_09
3912270	3912271	1		rd	2	NC252_09
3912353	3912354	1		rd	2	NC252_09
3914184	3914185	1		rd	2	NC252_09
3914662	3914663	1		rd	2	NC252_09
3914995	3915000	5		rd	2	NC252_09
3917615	3917616	1		rd	2	NC252_09
3919345	3919346	1		rd	2	NC252_09
3919981	3919982	1		rd	2	NC252_09
3920363	3920368	5		rd	2	NC252_09
3921155	3921156	1		rd	2	NC252_09
3921181	3921184	3		rd	2	NC252_09
3921211	3921212	1		rd	2	NC252_09
3923363	3923362	1		rd	2	NC252_09
3924098	3924099	1		rd	2	NC252_09
3924270	3924271	1		rd	2	NC252_09
3925347	3925348	1		rd	2	NC252_09
3926015	3926020	5		rd	2	NC252_09
3927088	3927089	1		rd	2	NC252_09
3927638	3927639	1		rd	2	NC252_09
3927915	3927916	1		rd	2	NC252_09
3928000	3928001	1		rd	2	NC252_09
3928311	3928314	3		rd	2	NC252_09
3928356	3928357	1		rd	2	NC252_09
3928616	3928617	1		rd	2	NC252_09
3928784	3928785	1		rd	2	NC252_09
3929773	3929775	2		rd	2	NC252_09
3930174	3930175	1		rd	2	NC252_09
3930174	3930175	1		rd	2	NC252_09
3930476	3930477	1		rd	2	NC252_09
3930538	3930539	1		rd	2	NC252_09
3930538	3930539	1		rd	2	NC252_09
3930538	3930539	1		rd	2	NC252_09
3930996	3930996	1		rd	2	NC252_09
3930996	3931000	3		rd	2	NC252_09
3931745	3931746	1		rd	2	NC252_09
3932386	3932387	1		rd	2	NC252_09
3932591	3932596	5		rd	2	NC252_09
3932715	3932720	5		rd	2	NC252_09
3932995	3933000	5		rd	2	NC252_09
3933000	3933001	1		rd	2	NC252_09
3933155	3933156	1		rd	2	NC252_09
3934002	3934003	1		rd	2	NC252_09
3934625	3934630	5		rd	2	NC252_09
3934796	3934797	1		rd	2	NC252_09
3934831	3934832	1		rd	2	NC252_09
3936395	3936396	1		rd	2	NC252_09
3938581	3938584	3		rd	2	NC252_09
3939688	3939690	2		rd	2	NC252_09
3940699	3940699	1		rd	2	NC252_09
3940702	3940703	1		rd	2	NC252_09
3940854	3940855	1		rd	2	NC252_09
Total						18

Figure 7.6 (cont)

Breakpoints		Size (bp)		MRU-WTR Cluster	SCG	Isolate
Start	End					
3940906	3940907	18		rd	1	IGTB423
3941383	3941384	1		NC	2	ARS7886
3941568	3941569	18		CC35079	2	CC35079
3942118	3942119	1		rd	2	NC2750_09
3942812	3942813	18		NC	2	NC2750_09
3943520	3943521	1		rd	2	NC2750_09
3943838	3943839	2		rd	2	NC2750_09
3943953	3943954	1		rd	2	NC2750_09
3944233	3944234	1		rd	2	NC2750_09
3944586	3944587	2		rd	2	NC2750_09
3945715	3945716	1		rd	2	NC2750_09
3946544	3946545	1		rd	2	NC2750_09
3946744	3946745	1		rd	2	NC2750_09
3947455	3947456	1		rd	2	NC2750_09
3948328	3948329	1		rd	2	NC2750_09
3948611	3948612	1		rd	2	NC2750_09
3949054	3949055	1		rd	2	NC2750_09
3949913	3949914	1		rd	2	NC2750_09
3951763	3951764	1		rd	2	NC2750_09
3952121	3952122	1		rd	2	NC2750_09
3952155	3952156	1		rd	2	NC2750_09
3953397	3953398	1		rd	2	NC2750_09
3953469	3953470	1		rd	2	NC2750_09
3958818	3958819	1		rd	2	NC2750_09
3959022	3959023	1		rd	2	NC2750_09
3959863	3959864	1		rd	2	NC2750_09
3960388	3960389	1		rd	2	NC2750_09
3962585	3962586	1		rd	2	NC2750_09
3962638	3962639	1		rd	2	NC2750_09
3963912	3963913	1		rd	2	NC2750_09
3964296	3964297	1		rd	2	NC2750_09
3965440	3965441	1		rd	2	NC2750_09
3965500	3965501	1		rd	2	NC2750_09
3966322	3966323	1		rd	2	NC2750_09
3967303	3967304	1		rd	2	NC2750_09
3967498	3967499	1		rd	2	NC2750_09
3968298	3968299	1		rd	2	NC2750_09
3970616	3970617	1		rd	2	NC2750_09
3971178	3971179	1		rd	2	NC2750_09
3972130	3972131	1		rd	2	NC2750_09
3972308	3972309	1		rd	2	NC2750_09
3972998	3972999	1		rd	2	NC2750_09
3973430	3973431	1		rd	2	NC2750_09
3976193	3976194	1		rd	2	NC2750_09
3976885	3976886	1		rd	2	NC2750_09
3977404	3977405	1		rd	2	NC2750_09
3977440	3977441	1		rd	2	NC2750_09
3982624	3982625	1		rd	2	NC2750_09
3983881	3983882	1		rd	2	NC2750_09
3983894	3983895	1		rd	2	NC2750_09
3984422	3984423	1		rd	2	NC2750_09
3985966	3985967	1		rd	2	NC2750_09
3990481	3990482	1		rd	2	NC2750_09
3991405	3991406	1		rd	2	NC2750_09
3991943	3991944	1		rd	2	NC2750_09
3998234	3998235	1		rd	2	NC2750_09
4000751	4000752	1		rd	2	NC2750_09
4001568	4001569	1		rd	2	NC2750_09
4001845	4001846	1		rd	2	NC2750_09
4001898	4001899	1		rd	2	NC2750_09
4004473	4004474	1		rd	2	NC2750_09
4004522	4004523	1		rd	2	NC2750_09
4006115	4006116	1		rd	2	NC2750_09
4007271	4007272	1		rd	2	NC2750_09
4010811	4010812	1		rd	2	NC2750_09
4013251	4013252	1		rd	2	NC2750_09
4018041	4018042	1		rd	2	NC2750_09
4018305	4018306	1		rd	2	NC2750_09
4023508	4023509	1		rd	2	NC2750_09
4024715	4024716	1		rd	2	NC2750_09
4027410	4027411	1		rd	2	NC2750_09
4027975	4027976	1		rd	2	NC2750_09
4029525	4029526	1		rd	2	NC2750_09
4030511	4030512	1		rd	2	NC2750_09
4031665	4031666	1		rd	2	NC2750_09
4033213	4033214	1		rd	2	NC2750_09
4033528	4033529	1		rd	2	NC2750_09
4033791	4033792	1		rd	2	NC2750_09
4036388	4036389	1		rd	2	NC2750_09
4036666	4036667	1		rd	2	NC2750_09
4037128	4037129	1		rd	2	NC2750_09
4037185	4037186	1		rd	2	NC2750_09
4037454	4037455	1		rd	2	NC2750_09
4037698	4037699	1		rd	2	NC2750_09
4038621	4038622	1		rd	2	NC2750_09
4039137	4039138	1		rd	2	NC2750_09
4040898	4040899	1		rd	2	NC2750_09
4041497	4041498	1		rd	2	NC2750_09
4042134	4042135	1		rd	2	NC2750_09
4044034	4044035	1		rd	2	NC2750_09
4049834	4049835	1		rd	2	NC2750_09
4049974	4049975	1		rd	2	NC2750_09
4054866	4054867	1		rd	2	NC2750_09
4058225	4058226	1		rd	2	NC2750_09
4058939	4058940	1		rd	2	NC2750_09
4059038	4059039	1		rd	2	NC2750_09
4060371	4060372	1		rd	2	NC2750_09
4060502	4060503	1		rd	2	NC2750_09
4060555	4060556	1		rd	2	NC2750_09
4061563	4061564	1		rd	2	NC2750_09
4061698	4061699	1		rd	2	NC2750_09
4062643	4062644	1		rd	2	NC2750_09
4063512	4063513	1		rd	2	NC2750_09
4064912	4064913	1		rd	2	NC2750_09
4067143	4067144	1		rd	2	NC2750_09
4067293	4067294	1		rd	2	NC2750_09
4067928	4067929	1		rd	2	NC2750_09
4068028	4068029	1		rd	2	NC2750_09
4071963	4071964	1		rd	2	NC2750_09
4071998	4071999	1		rd	2	NC2750_09
4071999	4072000	1		rd	2	NC2750_09
4072336	4072337	1		rd	2	NC2750_09
4073078	4073079	1		rd	2	NC2750_09
4075411	4075412	1		rd	2	NC2750_09
4076153	4076154	1		rd	2	NC2750_09
4076454	4076455	1		rd	2	NC2750_09
4076454	4076455	1		rd	2	NC2750_09
4076725	4076726	1		rd	2	NC2750_09
4076851	4076852	1		rd	2	NC2750_09
4076915	4076916	1		rd	2	NC2750_09
4080421	4080422	1		rd	2	NC2750_09
4081197	4081198	1		rd	2	NC2750_09
4081908	4081909	1		rd	2	NC2750_09
4082681	4082682	1		rd	2	NC2750_09
4082910	4082911	1		rd	2	NC2750_09
4083077	4083078	1		rd	2	NC2750_09
4083106	4083107	1		rd	2	NC2750_09
4083118	4083119	1		rd	2	NC2750_09
4085624	4085625	1		rd	2	NC2750_09
4086831	4086832	1		rd	2	NC2750_09
4086881	4086882	1		rd	2	NC2750_09
4087025	4087026	1		rd	2	NC2750_09
4087905	4087906	1		rd	2	NC2750_09
4087998	4087999	1		rd	2	NC2750_09
4088017	4088018	1		rd	2	NC2750_09
4088328	4088329	1		rd	2	NC2750_09
4088445	4088446	1		rd	2	NC2750_09
4088551	4088552	1		rd	2	NC2750_09
4091381	4091382	1		rd	2	NC2750_09
4091815	4091816	1		rd	2	NC2750_09
4093139	4093140	1		rd	2	NC2750_09
4093158	4093159	1		rd	2	NC2750_09
4094348	4094349	18		rd	2	NC2750_09
4094753	4094754	1		rd	2	NC2750_09
4097238	4097239	1		rd	2	NC2750_09
4097496	4097497	1		rd	2	NC2750_09
4100593	4100594	1		rd	2	NC2750_09
4100919	4100920	1		rd	2	NC2750_09
4101491	4101492	1		rd	2	NC2750_09
4101544	4101545	1		rd	2	NC2750_09
4102773	4102774	1		rd	2	NC2750_09
4106048	4106049	1		rd	2	NC2750_09
						Total

Figure 7.6 (cont)

Breakpoints		Size (bp)		SCG	Isolate
Start	End	MRU-WTR Cluster	SCG		
4106088	4106090	1	rd	1	IGTB423
4107373	4107374	2	NC	2	ARS7886
4107848	4107849	1	rd	2	CC53079
4108303	4108304	1	rd	2	NC250_09
4109810	4109811	1	rd	2	NC250_09
4110781	4110782	1	rd	2	NC250_09
4110961	4110964	2	rd	2	NC250_09
4112823	4112824	1	rd	2	NC250_09
4112962	4112963	1	rd	2	NC250_09
4113627	4113628	9	rd	2	NC250_09
4113998	4113999	1	rd	2	NC250_09
4114248	4114249	1	rd	2	NC250_09
4114764	4114765	1	rd	2	NC250_09
4114885	4114886	2	rd	2	NC250_09
4115637	4115638	1	rd	2	NC250_09
4116284	4116285	1	rd	2	NC250_09
4116624	4116625	1	rd	2	NC250_09
4117088	4117089	1	rd	2	NC250_09
4118394	4118395	1	rd	2	NC250_09
4120398	4120399	1	rd	2	NC250_09
4121103	4121104	1	rd	2	NC250_09
4121288	4121289	2	rd	2	NC250_09
4121484	4121485	1	rd	2	NC250_09
4122788	4122789	1	rd	2	NC250_09
4124688	4124689	1	rd	2	NC250_09
4127713	4127714	1	rd	2	NC250_09
4127972	4127973	1	rd	2	NC250_09
4128133	4128134	1	rd	2	NC250_09
4129910	4129911	2	rd	2	NC250_09
4130455	4130456	1	rd	2	NC250_09
4130508	4130509	1	rd	2	NC250_09
4132243	4132244	1	rd	2	NC250_09
4132753	4132754	1	rd	2	NC250_09
4133145	4133146	2	rd	2	NC250_09
4134078	4134079	1	rd	2	NC250_09
4135464	4135465	1	rd	2	NC250_09
4136228	4136229	1	rd	2	NC250_09
4138143	4138144	1	rd	2	NC250_09
4138688	4138689	1	rd	2	NC250_09
4139183	4139184	1	rd	2	NC250_09
4139318	4139319	2	rd	2	NC250_09
4140837	4140838	1	rd	2	NC250_09
4143524	4143525	1	rd	2	NC250_09
4144315	4144316	1	rd	2	NC250_09
4145829	4145830	1	rd	2	NC250_09
4145914	4145915	1	rd	2	NC250_09
4149753	4149754	1	rd	2	NC250_09
4151000	4151001	1	rd	2	NC250_09
4151558	4151559	1	rd	2	NC250_09
4155144	4155145	1	rd	2	NC250_09
4156205	4156206	2	rd	2	NC250_09
4156583	4156584	1	rd	2	NC250_09
4162444	4162445	1	rd	2	NC250_09
4162484	4162485	1	rd	2	NC250_09
4162738	4162739	5	rd	2	NC250_09
4164602	4164603	1	rd	2	NC250_09
4164774	4164775	1	rd	2	NC250_09
4164976	4164977	1	rd	2	NC250_09
4166047	4166048	3	rd	2	NC250_09
4168774	4168775	1	rd	2	NC250_09
4170964	4170965	1	rd	2	NC250_09
4170990	4170991	1	rd	2	NC250_09
4171318	4171319	1	rd	2	NC250_09
4173162	4173163	4	rd	2	NC250_09
4174164	4174165	1	rd	2	NC250_09
4175622	4175623	1	rd	2	NC250_09
4175678	4175679	1	rd	2	NC250_09
4176057	4176058	1	rd	2	NC250_09
4176513	4176514	1	rd	2	NC250_09
4177353	4177354	1	rd	2	NC250_09
4177356	4177357	1	rd	2	NC250_09
4178648	4178649	1	rd	2	NC250_09
4179503	4179504	1	rd	2	NC250_09
4180762	4180763	3	rd	2	NC250_09
4180805	4180806	2	rd	2	NC250_09
4181672	4181673	1	rd	2	NC250_09
4182528	4182529	1	rd	2	NC250_09
4182866	4182867	1	rd	2	NC250_09
4184045	4184046	1	rd	2	NC250_09
4185655	4185656	1	rd	2	NC250_09
4186126	4186127	1	rd	2	NC250_09
4190595	4190596	2	rd	2	NC250_09
4191275	4191276	2	rd	2	NC250_09
4191845	4191846	1	rd	2	NC250_09
4192213	4192214	1	rd	2	NC250_09
4193825	4193826	1	rd	2	NC250_09
4194718	4194719	1	rd	2	NC250_09
4195911	4195912	1	rd	2	NC250_09
4197046	4197047	1	rd	2	NC250_09
4197138	4197139	1	rd	2	NC250_09
4197574	4197575	1	rd	2	NC250_09
4197588	4197589	1	rd	2	NC250_09
4197708	4197709	1	rd	2	NC250_09
4199175	4199176	2	rd	2	NC250_09
4199664	4199665	1	rd	2	NC250_09
4201753	4201754	1	rd	2	NC250_09
4201877	4201878	1	rd	2	NC250_09
4202138	4202139	2	rd	2	NC250_09
4203393	4203394	1	rd	2	NC250_09
4203447	4203448	1	rd	2	NC250_09
4206258	4206259	1	rd	2	NC250_09
4208453	4208454	1	rd	2	NC250_09
4210568	4210569	1	rd	2	NC250_09
4210648	4210649	1	rd	2	NC250_09
4210905	4210906	1	rd	2	NC250_09
4212783	4212784	1	rd	2	NC250_09
4215410	4215411	1	rd	2	NC250_09
4215758	4215759	1	rd	2	NC250_09
4216094	4216095	1	rd	2	NC250_09
4217058	4217059	1	rd	2	NC250_09
4218953	4218954	1	rd	2	NC250_09
4219645	4219646	2	rd	2	NC250_09
4221408	4221409	1	rd	2	NC250_09
4222621	4222622	1	rd	2	NC250_09
4224475	4224476	1	rd	2	NC250_09
4226098	4226099	2	rd	2	NC250_09
4228013	4228014	1	rd	2	NC250_09
4230133	4230134	1	rd	2	NC250_09
4231225	4231226	1	rd	2	NC250_09
4231228	4231229	1	rd	2	NC250_09
4232323	4232324	1	rd	2	NC250_09
4234976	4234977	1	rd	2	NC250_09
4235999	4235999	1	rd	2	NC250_09
4236445	4236446	1	rd	2	NC250_09
4238697	4238698	1	rd	2	NC250_09
4241198	4241199	1	rd	2	NC250_09
4243561	4243562	1	rd	2	NC250_09
4245198	4245199	1	rd	2	NC250_09
4245619	4245620	1	rd	2	NC250_09
4246353	4246354	1	rd	2	NC250_09
4248073	4248074	4	rd	2	NC250_09
4249355	4249356	1	rd	2	NC250_09
4251733	4251734	5	rd	2	NC250_09
4252931	4252932	2	rd	2	NC250_09
4253198	4253199	1	rd	2	NC250_09
4253753	4253754	2	rd	2	NC250_09
4254334	4254335	1	rd	2	NC250_09
4254884	4254885	2	rd	2	NC250_09
4255388	4255389	2	rd	2	NC250_09
4257999	4257999	1	rd	2	NC250_09
4258334	4258335	1	rd	2	NC250_09
4260204	4260205	1	rd	2	NC250_09
4260993	4260994	1	rd	2	NC250_09
4261338	4261339	1	rd	2	NC250_09
4262166	4262167	1	rd	2	NC250_09
4262388	4262389	1	rd	2	NC250_09
4262508	4262509	1	rd	2	NC250_09
4262914	4262915	1	rd	2	NC250_09
4262998	4262999	1	rd	2	NC250_09
4264201	4264202	1	rd	2	NC250_09
4264383	4264384	1	rd	2	NC250_09
4265123	4265124	1	rd	2	NC250_09
Total					1

Breakpoints		Size (bp)		MIRU-VNTR Cluster	SCG	Isolate
Start	End					
4266652	4266653	1		rd	1	IGTB423
4268740	4268741	1		NC	2	ARS7896
4268805	4268870	1		rd	2	CC53079
4269335	4269340	1		rd	2	NC250_09
4269692	4269693	1		rd	2	NC250_09
4269763	4269766	1		rd	2	NC250_09
4272233	4272234	1		rd	2	NC250_09
4274103	4274103	1		rd	2	NC250_09
4274139	4274140	1		rd	2	NC250_09
4278965	4278966	1		rd	2	NC250_09
4280995	4281000	2		rd	2	NC250_09
4283073	4283074	2		rd	2	NC250_09
4283139	4283140	1		rd	2	NC250_09
4286995	4287000	1		rd	2	NC250_09
4287995	4288000	2		rd	2	NC250_09
4289033	4289034	1		rd	2	NC250_09
4292277	4292278	1		rd	2	NC250_09
4297259	4297260	1		rd	2	NC250_09
4298375	4298376	1		rd	2	NC250_09
4298995	4298996	1		rd	2	NC250_09
4300763	4300764	1		rd	2	NC250_09
4301724	4301725	1		rd	2	NC250_09
4302953	4302954	1		rd	2	NC250_09
4303318	4303319	1		rd	2	NC250_09
4303498	4303499	1		rd	2	NC250_09
4303538	4303539	1		rd	2	NC250_09
4304393	4304394	1		rd	2	NC250_09
4304623	4304624	1		rd	2	NC250_09
4304755	4304756	1		rd	2	NC250_09
4305063	4305064	1		rd	2	NC250_09
4307248	4307249	1		rd	2	NC250_09
4307385	4307386	1		rd	2	NC250_09
4309133	4309134	5		rd	2	NC250_09
4309494	4309495	1		rd	2	NC250_09
4310743	4310744	1		rd	2	NC250_09
4311677	4311678	1		rd	2	NC250_09
4313513	4313514	1		rd	2	NC250_09
4315268	4315269	6		rd	2	NC250_09
4315703	4315704	1		rd	2	NC250_09
4317373	4317374	1		rd	2	NC250_09
4317425	4317426	1		rd	2	NC250_09
4317903	4317904	1		rd	2	NC250_09
4318374	4318375	1		rd	2	NC250_09
4320592	4320593	2		rd	2	NC250_09
4323149	4323150	2		rd	2	NC250_09
4323354	4323355	1		rd	2	NC250_09
4323695	4323696	1		rd	2	NC250_09
4324383	4324384	2		rd	2	NC250_09
4324940	4324941	1		rd	2	NC250_09
4325153	4325154	1		rd	2	NC250_09
4326582	4326583	1		rd	2	NC250_09
4326634	4326635	1		rd	2	NC250_09
4326805	4326806	2		rd	2	NC250_09
4327225	4327226	1		rd	2	NC250_09
4330330	4330331	1		rd	2	NC250_09
4332293	4332294	1		rd	2	NC250_09
4333156	4333157	1		rd	2	NC250_09
4334828	4334829	1		rd	2	NC250_09
4336095	4336096	1		rd	2	NC250_09
4336718	4336719	1		rd	2	NC250_09
4339728	4339729	1		rd	2	NC250_09
4340398	4340399	5		rd	2	NC250_09
4341012	4341013	1		rd	2	NC250_09
4341782	4341783	1		rd	2	NC250_09
4344393	4344394	1		rd	2	NC250_09
4345613	4345614	1		rd	2	NC250_09
4346013	4346014	1		rd	2	NC250_09
4346054	4346055	1		rd	2	NC250_09
4346368	4346369	1		rd	2	NC250_09
4346492	4346493	1		rd	2	NC250_09
4346773	4346774	5		rd	2	NC250_09
4350093	4350094	1		rd	2	NC250_09
4350308	4350309	2		rd	2	NC250_09
4352038	4352039	1		rd	2	NC250_09
4352663	4352664	2		rd	2	NC250_09
4353986	4353987	1		rd	2	NC250_09
4355488	4355489	1		rd	2	NC250_09
4355493	4355494	4		rd	2	NC250_09
4357995	4357996	1		rd	2	NC250_09
4358350	4358351	1		rd	2	NC250_09
4358594	4358595	1		rd	2	NC250_09
4358883	4358884	1		rd	2	NC250_09
4358978	4358979	1		rd	2	NC250_09
4359073	4359074	1		rd	2	NC250_09
4359133	4359134	10		rd	2	NC250_09
4359135	4359136	30		rd	2	NC250_09
4359169	4359170	18		rd	2	NC250_09
4359170	4359171	30		rd	2	NC250_09
4359216	4359217	1		rd	2	NC250_09
4359753	4359754	1		rd	2	NC250_09
4360823	4360824	1		rd	2	NC250_09
4362435	4362436	1		rd	2	NC250_09
4363085	4363086	1		rd	2	NC250_09
4364973	4364974	1		rd	2	NC250_09
4366812	4366813	1		rd	2	NC250_09
4367123	4367124	1		rd	2	NC250_09
4368380	4368381	1		rd	2	NC250_09
4369344	4369345	1		rd	2	NC250_09
4369372	4369373	1		rd	2	NC250_09
4369523	4369524	1		rd	2	NC250_09
4369590	4369591	1		rd	2	NC250_09
4369645	4369646	1		rd	2	NC250_09
4372903	4372904	1		rd	2	NC250_09
4373363	4373364	1		rd	2	NC250_09
4374643	4374644	1		rd	2	NC250_09
4375233	4375234	1		rd	2	NC250_09
4375463	4375464	1		rd	2	NC250_09
4375788	4375789	1		rd	2	NC250_09
4377524	4377525	2		rd	2	NC250_09
4381346	4381347	1		rd	2	NC250_09
4382333	4382334	1		rd	2	NC250_09
4383144	4383145	5		rd	2	NC250_09
4383393	4383394	1		rd	2	NC250_09
4383556	4383557	1		rd	2	NC250_09
4383962	4383963	1		rd	2	NC250_09
4384714	4384715	1		rd	2	NC250_09
4386634	4386635	1		rd	2	NC250_09
4386680	4386681	1		rd	2	NC250_09
4387125	4387126	1		rd	2	NC250_09
4387433	4387434	1		rd	2	NC250_09
4387485	4387486	1		rd	2	NC250_09
4387853	4387854	1		rd	2	NC250_09
4388995	4388996	1		rd	2	NC250_09
4389478	4389479	1		rd	2	NC250_09
4390105	4390106	3		rd	2	NC250_09
4390882	4390883	3		rd	2	NC250_09
4392523	4392524	1		rd	2	NC250_09
4392903	4392904	1		rd	2	NC250_09
4394558	4394559	1		rd	2	NC250_09
4394893	4394894	1		rd	2	NC250_09
4396085	4396086	1		rd	2	NC250_09
4397915	4397916	1		rd	2	NC250_09
4402343	4402344	2		rd	2	NC250_09
4404728	4404729	1		rd	2	NC250_09
4407823	4407824	1		rd	2	NC250_09
4407874	4407875	1		rd	2	NC250_09
4408944	4408945	8		rd	2	NC250_09
4409493	4409494	1		rd	2	NC250_09
4409748	4409749	1		rd	2	NC250_09
Total		1811				
4266652	4266653	1		rd	1	IGTB423
4268740	4268741	1		NC	2	ARS7896
4268805	4268870	1		rd	2	CC53079
4269335	4269340	1		rd	2	NC250_09
4269692	4269693	1		rd	2	NC250_09
4269763	4269766	1		rd	2	NC250_09
4272233	4272234	1		rd	2	NC250_09
4274103	4274103	1		rd	2	NC250_09
4274139	4274140	1		rd	2	NC250_09
4278965	4278966	1		rd	2	NC250_09
4280995	4281000	2		rd	2	NC250_09
4283073	4283074	2		rd	2	NC250_09
4283139	4283140	1		rd	2	NC250_09
4286995	4287000	1		rd	2	NC250_09
4287995	4288000	2		rd	2	NC250_09
4289033	4289034	1		rd	2	NC250_09
4292277	4292278	1		rd	2	NC250_09
4297259	4297260	1		rd	2	NC250_09
4298375	4298376	1		rd	2	NC250_09
4298995	4298996	1		rd	2	NC250_09
4300763	4300764	1		rd	2	NC250_09
4301724	4301725	1		rd	2	NC250_09
4302953	4302954	1		rd	2	NC250_09
4303318	4303319	1		rd	2	NC250_09
4303498	4303499	1		rd	2	NC250_09
4303538	4303539	1		rd	2	NC250_09
4304393	4304394	1		rd	2	NC250_09
4304623	4304624	1		rd	2	NC250_09
4304755	4304756	1		rd	2	NC250_09
4305063	4305064	1		rd	2	NC250_09
4307248	4307249	1		rd	2	NC250_09
4307385	4307386	1		rd	2	NC250_09
4309133	4309134	5		rd	2	NC250_09
4309494	4309495	1		rd	2	NC250_09
4310743	4310744	1		rd	2	NC250_09
4311677	4311678	1		rd	2	NC250_09
4313513	4313514	1		rd	2	NC250_09
4315268	4315269	6		rd	2	NC250_09
4315703	4315704	1		rd	2	NC250_09
4317373	4317374	1		rd	2	NC250_09
4317425	4317426	1		rd	2	NC250_09
4317903	4317904	1		rd	2	NC250_09
4318374	4318375	1		rd	2	NC250_09
4320592	4320593	2		rd	2	NC250_09
4323149	4323150	2		rd	2	NC250_09
4323354	4323355	1		rd	2	NC250_09
4323695	4323696	1		rd	2	NC250_09
4324383	4324384	2		rd	2	NC250_09
4324940	4324941	1		rd	2	NC250_09
4325153	4325154	1		rd	2	NC250_09
432658						

Figure S7.7

Breakpoints		Size (B)		SV Type	VMU-WFE Cluster	DCG	VMU	Status
Start	End	Size (B)	SV Type					
102544	102556	439	NS					
20787	20789	127	NS					
28843	28845	134	NS					
28853	28855	142	NS					
69257	70624	926	DEL					
72995	72997	128	NS					
79558	82661	480	DEL					
79560	83594	2944	DEL					
79562	83685	365	DEL					
79639	83027	4408	DEL					
79953	83059	5176	DEL					
81786	82325	4429	DEL					
87940	88373	243	DEL					
113759	114409	560	DEL					
148847	151208	1362	DEL					
149853	150919	5028	DEL					
149854	150915	807	DEL					
150249	150247	243	NS					
150489	150481	542	NS					
150893	150903	180	NS					
150903	150909	180	NS					
150909	150905	162	NS					
150949	150938	180	NS					
164499	163739	760	DEL					
167923	168226	273	DEL					
168468	168468	822	DEL					
171457	171776	322	DEL					
221753	221752	130	NS					
233657	149584	2377	DEL					
133670	133887	2217	DEL					
133768	146659	2562	DEL					
134665	134657	712	DEL					
134973	133846	1335	DEL					
135074	139228	2544	DEL					
136033	136045	2700	DEL					
136487	136239	1489	DEL					
136305	136634	2783	DEL					
136350	137224	1332	DEL					
136364	136631	2519	DEL					
136609	136705	2092	DEL					
136629	137736	1147	DEL					
136668	137450	782	DEL					
136764	138844	2170	DEL					
137796	138631	835	DEL					
140393	140093	300	DEL					
140423	140423	822	DEL					
150960	150962	119	NS					
363533	363502	330	DEL					
363746	362977	1211	DEL					
363754	362060	246	DEL					
363779	362058	243	DEL					
362437	362647	210	DEL					
383676	383681	137	NS					
404473	404475	138	NS					
426952	427108	156	DEL					
426959	427102	156	DEL					
426955	427111	156	DEL					
427138	427136	145	NS					
433173	433178	606	DEL					
433779	433684	2605	DEL					
437926	437938	126	NS					
509995	509909	127	NS					
623933	624096	5003	DEL					
623444	624475	313	DEL					
623224	624883	1159	DEL					
623369	623384	120	DEL					
623637	623832	190	DEL					
623775	624393	612	DEL					
672665	674479	4472	DEL					
672018	675389	3371	DEL					
671385	672534	540	DEL					
674995	675232	237	DEL					
674155	674498	340	DEL					
680365	684448	1566	DEL					
721485	723187	139	NS					
742629	742636	562	NS					
742633	742635	533	NS					
742631	742639	475	NS					
744475	746825	441	DEL					
745180	748449	238	DEL					
746380	746382	100	NS					
747292	747645	350	DEL					
83268	833038	252	DEL					
83595	836146	1146	DEL					
836186	836899	713	DEL					
836257	837886	978	DEL					
836353	836499	136	DEL					
837185	837958	780	DEL					
838775	839465	697	DEL					
838776	840696	920	DEL					
838833	840626	833	DEL					
839140	840203	153	DEL					
83944	840623	736	DEL					
837999	837963	139	NS					
880554	880429	875	DEL					
887013	880375	1862	INV					
887013	880375	1862	INV					
887000	880375	1375	INV					
887763	889220	238	INV					
888762	889202	238	INV					
888762	880375	1601	INCOMPLEX					
888762	880375	1601	INCOMPLEX					
888762	880375	1601	INCOMPLEX					
888762	880375	1601	INCOMPLEX					
888762	880375	1601	INCOMPLEX					
888762	880375	1601	INCOMPLEX					
888762	880375	1601	INCOMPLEX					
888762	880375	1601	INCOMPLEX					
888762	880375	1601	INCOMPLEX					
888762	880375	1601	INCOMPLEX					
888762	880375	1601	INCOMPLEX					
888762	880375	1601	INCOMPLEX					
888762	880375	1601	INCOMPLEX					
888762	880375	1601	INCOMPLEX					
888762	880375	1601	INCOMPLEX					
888762	880375	1601	INCOMPLEX					
888762	880375	1601	INCOMPLEX					
888762	880375	1601	INCOMPLEX					
888762	880375	1601	INCOMPLEX					
888762	880375	1601	INCOMPLEX					
888762	880375	1601	INCOMPLEX					
888762	880375	1601	INCOMPLEX					
888762	880375	1601	INCOMPLEX					
888762	880375	1601	INCOMPLEX					
888762	880375	1601	INCOMPLEX					
888762	880375	1601	INCOMPLEX					
888762	880375	1601	INCOMPLEX					
888762	880375	1601	INCOMPLEX					
888762	880375	1601	INCOMPLEX					
888762	880375	1601	INCOMPLEX					
888762	880375	1601	INCOMPLEX					
888762	880375	1601	INCOMPLEX					
888762	880375	1601	INCOMPLEX					
888762	880375	1601	INCOMPLEX					
888762	880375	1601	INCOMPLEX					
888762	880375	1601	INCOMPLEX					
888762	880375	1601	INCOMPLEX					
888762	880375	1601	INCOMPLEX					
888762	880375	1601	INCOMPLEX					
888762	880375	1601	INCOMPLEX					
888762	880375	1601	INCOMPLEX					
888762	880375	1601	INCOMPLEX					
888762	880375	1601	INCOMPLEX					
888762	880375	1601	INCOMPLEX					
888762	880375	1601	INCOMPLEX					
888762	880375	1601	INCOMPLEX					
888762	880375	1601						





Figure S7.7 (cont)



Figure S7.7 (cont)

Breakpoints		Size (bp)	SV Type	Isolate			
Start	End			SV	MIRU-VNTR Cluster		
		3948826	39488472	646	DEL	nd	nd
		3948827	39488494	627	DEL	nd	nd
		3948830	39488478	644	DEL	nd	nd
		3948833	39488481	651	DEL	nd	nd
		3948831	39488479	648	DEL	nd	nd
		3948811	39488487	656	DEL	nd	nd
		3948833	39488485	652	DEL	nd	nd
		3948833	39488493	660	DEL	nd	nd
		3948834	39488489	655	DEL	nd	nd
		3948835	39488483	648	DEL	nd	nd
		3948847	39488471	624	DEL	nd	nd
		3948851	39488489	638	DEL	nd	nd
		3948875	39488489	604	DEL	nd	nd
		3949052	39488883	831	DEL	nd	nd
		3949052	39488895	833	DEL	nd	nd
		3949074	39488478	604	DEL	nd	nd
		3949080	39488477	597	DEL	nd	nd
		3949081	39488793	811	DEL	nd	nd
		3952928	3954226	1298	DEL	nd	nd
		3955477	3956117	640	DEL	nd	nd
		3972231	3972231	111	INS	nd	nd
		4002012	4002014	112	INS	nd	nd
		4031779	4031808	139	DEL	nd	nd
		4031885	4032250	564	DEL	nd	nd
		4031857	4031862	135	DEL	nd	nd
		4031508	4031843	135	DEL	nd	nd
		4031508	4031844	136	DEL	nd	nd
		4031516	4031850	115	DEL	nd	nd
		4031529	4031867	118	DEL	nd	nd
		4031774	4031752	738	DEL	nd	nd
		4031834	4031764	670	DEL	nd	nd
		4031810	4031767	697	DEL	nd	nd
		4048977	4048979	108	INS	nd	nd
		4052983	4053427	444	DEL	nd	nd
		4057489	4057555	143	INS	nd	nd
		4059748	4059817	189	DEL	nd	nd
		4059601	4059491	671	DEL	nd	nd
		4059509	4059494	115	DEL	nd	nd
		4188257	4188385	1108	DEL	nd	nd
		4324796	4325066	529	DEL	nd	nd
		4337628	4337628	142	INS	nd	nd
		4356484	4356715	420	DEL	nd	nd
		4357053	4357085	110	INS	nd	nd
		4370433	4371241	2808	DEL	nd	nd
		4382455	4382575	540	DEL	nd	nd
		4390137	4390138	131	INS	nd	nd
		4400902	4400549	642	DEL	nd	nd
		4407515	4408061	131	DEL	nd	nd
		4407921	4408051	230	DEL	nd	nd
Total						nd	nd

**Figure S7.7** - List of SV types found among the 75 clinical isolates group using the SVMerge pipeline and excluding copy number gain hits. SV type includes: deletions (DEL); completely (INSI) and incompletely (INS) reconstructed insertions; simple inversions (INV) and complex inversions (INVCOMPLEX); deletions plus insertions (DELINS); and, inversions plus deletions (INVDEL) or plus insertions (INVINS). Black squares are indicative of SV detection. MIRU-VNTR cluster indicates the 24-loci MIRU-VNTR cluster of any given isolate, except if non-clustered (NC) or not determined (nd). Line and column totals indicate total column/line count of SVs.

<sup>a</sup> did not map on RGTB423

<sup>b</sup> End not used for mapping

<sup>c</sup> Mapping of the IS1540 5' end yielded multiple hits per strain and highly variable, for this reason it was omitted.

did not map on HCC1095\_10

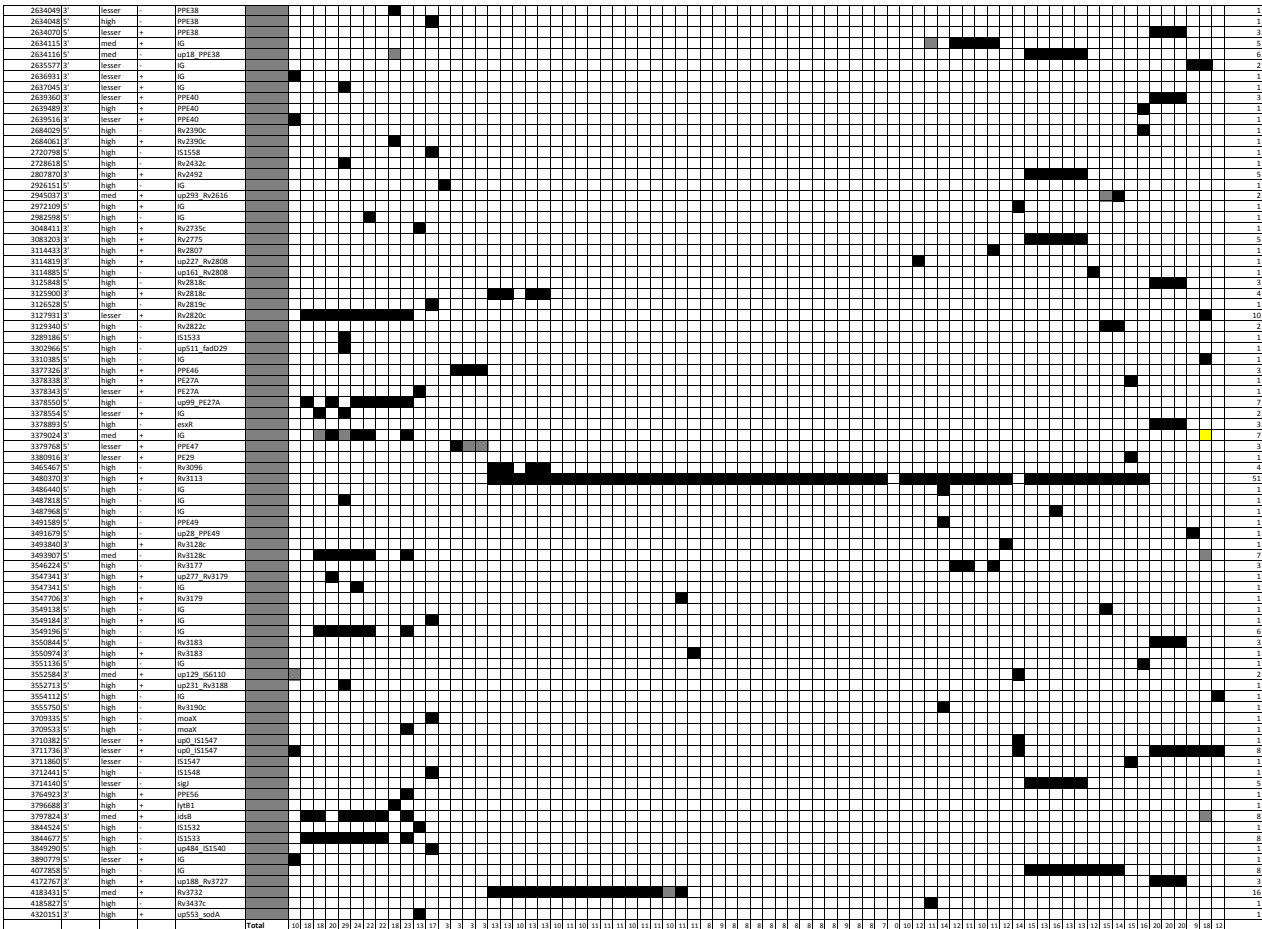
<sup>e</sup> did not map on X122

<sup>f</sup> did not map on CCDC5079

<sup>g</sup> did not map on ARS9427

<sup>c</sup> did not map on IHMT194\_11 and ARS9427

Position	Mapped End	Confidence	Chain	ORF	Isolate		Total
					SOS	SOS with Chain	
15951	5'	high	+	up463.dnaN	NC	8	
1873	3'	high	+	up177.dnaN	NC	1	
1877	3'	high	+	up175.dnaN	NC	1	
65238	5'	high	+	ru0061	NC	1	
401263	5'	high	+	ru0336	NC	8	
475210	5'	terseer	+	ru0395	NC	1	
483295	5'	high	+	rmn52	NC	1	
483585	5'	high	+	rmn55	NC	33	
607626	5'	high	+	ru0515	NC	8	
613995	3'	high	+	gagP	NC	1	
798653	3'	high	+	up180.ru0608	NC	2	
850083	3'	high	+	IG	NC	1	
850176	3'	high	+	up136.PPE12	NC	1	
850535	3'	high	+	IG	NC	1	
888786	5'	terseer	+	up150.ru0794c	NC	9	
888786	3'	terseer	+	up235.Gu110	NC	1	
888790	3'	terseer	+	up154.ru0794c	NC	1	
888927	5'	terseer	+	up94.Gu110	NC	3	
888993	5'	terseer	+	up28.Gu110	NC	1	
889033	5'	terseer	+	up179.ru0794c	NC	1	
889033	3'	high	+	up385.ru0794c	NC	16	
889033	5'	terseer	+	up13.G1547	NC	1	
890499	5'	terseer	+	up12.G1547	NC	8	
891080	5'	high	+	G1547	NC	1	
901401	5'	high	+	up180.ru0807	NC	1	
901406	5'	high	+	up229.ru0807	NC	1	
901574	3'	high	+	up61.ru0807	NC	1	
911887	3'	terseer	+	IG	NC	1	
912201	5'	med	+	IG	NC	51	
937114	3'	high	+	gag	NC	2	
985511	5'	high	+	up40.ru0887c	NC	1	
1026813	3'	high	+	G1554	NC	1	
1026903	5'	high	+	up10.G1554	NC	1	
1075885	5'	high	+	ru0963c	NC	1	
1075947	5'	high	+	ru0963c	NC	1	
1084088	3'	med	+	IG	NC	2	
1085603	5'	terseer	+	up309.mpaA	NC	1	
1101615	3'	high	+	up188.ru0986	NC	1	
1175581	3'	high	+	up142.ru1052	NC	1	
1175639	5'	high	+	up309.ru1051c	NC	1	
1262964	3'	high	+	PPE16	NC	6	
1481528	3'	high	+	ru1319c	NC	42	
1481528	3'	high	+	ru1319c	NC	3	
1533888	3'	high	+	IG	NC	1	
1541562	3'	high	+	IG	NC	2	
1543950	5'	high	+	up44.Gu110	NC	1	
1543950	5'	high	+	ru1371	NC	9	
1543968	5'	high	+	ru1372	NC	1	
1657012	5'	high	+	gagP	NC	1	
1694444	5'	high	+	ru1505c	NC	1	
1694469	5'	high	+	ru1504c	NC	1	
1695399	3'	high	+	ru1505c	NC	1	
1695518	5'	high	+	ru1505c	NC	1	
1710668	3'	high	+	up67.ru1519	NC	1	
1712384	5'	high	+	gag025	NC	1	
1761421	5'	high	+	ru1556	NC	1	
1890164	5'	terseer	+	gag	NC	1	
1895414	5'	med	+	up112.ru1669	NC	16	
1895603	5'	high	+	up108.ru1668c	NC	1	
1896054	5'	med	+	ru1669	NC	2	
1906425	5'	high	+	ru1682	NC	4	
1915313	5'	high	+	up117.gpi	NC	1	
1928049	5'	high	+	ru1702c	NC	1	
1950764	3'	terseer	+	ru1724c	NC	1	
1957891	3'	high	+	gag02	NC	1	
1981599	3'	high	+	IG	NC	1	
1986621	3'	terseer	+	ru1754c	NC	1	
1986626	3'	terseer	+	ru1754c	NC	50	
1986627	3'	terseer	+	ru1754c	NC	1	
1986639	3'	terseer	+	ru1754c	NC	1	
1987082	3'	high	+	gag0	NC	9	
1987456	5'	terseer	+	gag0	NC	1	
1987466	3'	terseer	+	gag0	NC	1	
1987699	3'	terseer	+	gag0	NC	3	
1987703	3'	terseer	+	up07.gpi0	NC	1	
1989025	3'	terseer	+	gag1	NC	1	
1989057	3'	terseer	+	gag1	NC	1	
1989079	3'	terseer	+	gag1	NC	17	
1992736	3'	high	+	up117.ru1760	NC	1	
1992736	5'	terseer	+	up159.ru1759c	NC	1	
1996101	3'	terseer	+	IG	NC	2	
1997455	3'	terseer	+	IG	NC	2	
1997623	5'	high	+	ru1765c	NC	1	
1997623	5'	high	+	ru1765c	NC	2	
1998469	3'	high	+	ru1765c	NC	1	
1998599	3'	high	+	G89	NC	1	
1998622	5'	terseer	+	G89	NC	8	
1998787	3'	high	+	G89	NC	1	
1998792	5'	med	+	G89	NC	2	
1998809	5'	terseer	+	G89	NC	33	
1998837	5'	terseer	+	G89	NC	1	
1999057	3'	high	+	G89	NC	1	
1999703	3'	high	+	G89	NC	1	
1999884	3'	high	+	ru1766	NC	1	
2010911	5'	high	+	gag144	NC	1	
2018723	3'	high	+	up436.lpp1	NC	1	
2018790	5'	terseer	+	up369.lpp1	NC	3	
2018808	5'	high	+	IG	NC	1	
2018895	5'	high	+	IG	NC	1	
2019173	3'	terseer	+	lpp1	NC	3	
2040443	3'	high	+	PPE28	NC	1	
2040586	5'	terseer	+	PPE28	NC	1	
2040717	3'	terseer	+	PPE28	NC	1	
2041235	3'	high	+	PPE28	NC	1	
2041743	3'	terseer	+	IG	NC	1	
2046688	3'	high	+	IG	NC	1	
2047367	5'	high	+	up18.ru1804c	NC	5	
2103393	5'	terseer	+	PPE34	NC	1	
2103393	5'	terseer	+	PPE34	NC	5	
2103402	3'	high	+	PPE34	NC	1	
2103462	3'	terseer	+	PPE34	NC	1	
2103462	3'	med	+	PPE34	NC	6	
2103474	5'	terseer	+	PPE34	NC	1	
2103666	5'	high	+	PPE34	NC	1	
2105918	5'	terseer	+	PPE34	NC	1	
2105987	3'	high	+	PPE34	NC	1	
2106112	3'	high	+	PPE34	NC	1	
2106308	3'	high	+	PPE34	NC	1	
2180842	5'	high	+	ru1928c	NC	3	
2180857	3'	high	+	ru1928c	NC	1	
2215941	3'	high	+	up213.ru1977	NC	1	
2219788	3'	high	+	ru1977	NC	1	
2245298	3'	high	+	ru2000	NC	3	
2262012	3'	terseer	+	ru2015c	NC	2	
2262179	5'	high	+	ru2015c	NC	1	
2262184	5'	high	+	ru2015c	NC	2	
2263020	5'	high	+	ru2016	NC	1	
2263160	5'	terseer	+	up266.ru2016	NC	1	
2263624	5'	high	+	ru2016	NC	8	
2263779	3'	terseer	+	ru2016	NC	1	
2265112	3'	terseer	+	IG	NC	3	
2266085	3'	high	+	ru2019	NC	2	
2266164	5'	terseer	+	ru2019	NC	1	
2266240	5'	terseer	+	ru2019	NC	3	
2289268	5'	high	+	up27.pcaA	NC	2	
2334671	3'	high	+	up180.ru2078	NC	1	
2334685	3'	high	+	up174.ru2078	NC	2	
2364762	5'	high	+	ru2104c	NC	1	
2365414	5'	high	+	up591.PE22	NC	1	
2366893	5'	med	+	IG	NC	9	
2366897	3'	terseer	+	IG	NC	5	
2367205	5'	med	+	IG	NC	4	
2367206	5'	terseer	+	IG	NC	1	
2367678	3'	high	+	up38.PPE36	NC	3	
2367678	3'	high	+	IG	NC	1	
2386213	3'	high	+	up80.ru2125	NC	1	
2430113	3'	high	+	up259.ru2166c	NC	1	
2432904	5'	high	+	G1558	NC	1	
2442347	5'	high	+	ru2180c	NC	2	
2456838	5'	med	+	up95.trpD	NC	33	
2550034	5'	high	+	up192.ru2280	NC	1	
2555928	5'	high	+	up12.ru2280c	NC	2	
2559501	5'	high	+	ru2286c	NC	1	
2582457	5'	high	+	up159.ru2309c	NC	13	
2584431	3'	high	+	up18.ru23112	NC	1	
2608661	5'	high	+	up135.cysK1	NC	1	
2610660	3'	high	+	ru2336	NC	1	
2610897	5'	terseer	+	ru2336	NC	1	
2628462	5'	high	+	gagC	NC	4	
2628681	3'	terseer	+	gagC	NC	1	
2630571	5'	high	+	gagA	NC	1	
2633549	5'	terseer	+	PPE38	NC	1	
2633840	3'	high	+	PPE38	NC	1	
2634021	3'	terseer	+	PPE38	NC	1	
2634049	5'	terseer	+	PPE38	NC	1	



**Figure S7.9** – Mapped positions of IS6110 found across the genomes of the 75 analyzed *M. tuberculosis* clinical isolates in relation to *M. tuberculosis* H37Rv. Each mapped position shown refers to a IS6110 end and from which the genomic position of insertion was deduced, referred on the Mapped End column. Chain column shows the chain coding for IS6110 copy in context and consequently, its orientation. Confidence column corresponds to the quality/confidence level classification explained in the Materials and Methods section. ORF column shows: the affected ORF in case of an intragenic insertion site; intergenic if the site is intergenic and mapped IS is not on the proper orientation to exert a putative upregulatory effect on an ORF located downstream of the IS 3' end; or, the prefix up indicating that the mapped IS is upstream and in the same orientation of a downstream ORF, followed by a number indicating the distance to the downstream ORF and, followed by the ORF designation, gene or feature designation. Black squares indicate IS6110 copies mapped at both 5' and 3' end; grey squares indicate IS6110 copies that only the mapped end indicated in the Mapped End column was mapped; and, yellow squares indicate IS6110 copies on which the only mapped end is the other end than the one indicated in the Mapped End column. MIRU-VNTR cluster indicates the 24-loci MIRU-VNTR cluster of any given isolate, except if non-clustered (NC) or not determined (nd). Column and line totals account for the number of IS6110 copies mapped on each line and column, respectively. Isolates highlighted in red and blue belong to L3isoB4 and Q1 clades, respectively.

**Table S7.1-** Number of mutations categorized by structural and functional effect type found along specified branches of the Lisboa3 subtree.

Lisboa 3				Tree Branch						
				A-B	B-B1	B-C	C-D	D-D1	D-E	E-E1
Resistance acquisition <sup>a</sup>				INH	RIF			RIF	RIF	EMB
				STP	EMB					PZA
					FQ					FQ
					KAN					KAN
					AMK					
					CAP					
Structural type										
SNPs				23	237	2	2	80	48	187
INDELS					78			3		5
Mutational Effect										
Synonymous				10	95	2	2	31	20	73
Missense				12	113			44	23	94
Nonsense					3				1	2
Frameshift					38			3		
Start Lost					1			1		1
Stop Lost					1					
Codon Deletion					6					2
Codon Insertion					3					
Codon Change + Codon Deletion					3					1
Codon Change + Codon Insertion					3					
rRNA mutation										
Intergenic				1	49			4	4	19

<sup>a</sup>INH, isoniazid; HL, High-level resistance; STP, streptomycin; RIF, rifampicin; EMB, ethambutol; PZA, pyrazinamide; KAN, kanamycin; AMK; amikacin; CAP, capreomycin; FQ, fluoroquinolone.

**Table S7.2** – Number of mutations categorized by structural and functional effect type found along specified branches of the Q1 subtree.

Q1	Tree Branch					
	A-B	B-C	C-D	D-E	E-F	F-G
<b>Resistance acquisition<sup>a</sup></b>	INH	INH (HL) STP	RIF EMB PZA		KAN AMK CAP	FQ
<b>Structural type</b>						
SNPs	36	38	5	1	1	1
INDELS		4	1			2
<b>Mutational Effect</b>						
Synonymous	11	17				
Missense	19	21	5	1		2
Nonsense						
Frameshift		3				
Start Lost						
Stop Lost						
Codon Deletion						
Codon Insertion						
Codon Change + Codon Deletion						
Codon Change + Codon Insertion						
rRNA mutation					1	
Intergenic	6	3	1			1

<sup>a</sup>INH, isoniazid; HL, High-level resistance; STP, streptomycin; RIF, rifampicin; EMB, ethambutol; PZA, pyrazinamide; KAN, kanamycin; AMK; amikacin; CAP, capreomycin; FQ, fluoroquinolone.



**Table S7.3** - Mutations found to be acquired along node-delimited branches in the Lisboa3 subtree. Position, Reference Sequence and Mutated Sequence are derived from the VCF format.

Branch	Position	Reference Sequence	Mutated Sequence	Type	Codon Change <sup>a</sup>	Affected ORF	Mutation
A-B	405812 T	C	Missense	atA/atG	Rv0338c	I10M	
	740561 C	T	Synonymous	ccG/ccA	lipG	P193	
	781687 A	G	Missense	aAg/aGg	rpsL	K43R	
	1057412 A	G	Missense	cTa/tCa	Rv0947c	L40P	
	1153137 G	A	Missense	Gcg/Acg	kdpA	A376T	
	1673425 C	T	Intergenic				
	1674481 T	G	Missense	Tcg/Gcg	inhA	S94A	
	1706439 G	A	Synonymous	Ctg/Ttg	Rv1514c	L53	
	1885422 C	T	Missense	tCg/tTg	pkS8	S1240L	
	2123086 C	A	Synonymous	ccG/ccT	lldD2	P22	
	2255294 T	C	Missense	aTc/aCc	otsB1	I1098T	
	2264189 C	T	Synonymous	caC/caT	Rv2017	H64	
	2817383 C	T	Synonymous	ctG/ctA	accD1	L364	
	3256167 C	T	Synonymous	taC/taT	ppsC	Y161	
	3385982 C	T	Synonymous	cgG/cgA	Rv3026c	R32	
	3431653 G	A	Missense	Gat/Aat	Rv3067	D76N	
	3434043 G	A	Missense	Gtg/Atg	ccrB	V118M	
	3540581 A	G	Synonymous	caT/caC	hpx	H55	
	3544710 T	C	Synonymous	ccA/ccG	rmeT	P197	
	3730361 C	T	Missense	gGc/gAc	PPE54	G2192D	
	3883690 G	A	Missense	Gcg/Acg	Rv3466	A56T	
	4034894 G	A	Synonymous	ccG/ccA	lpqF	P181	
	4104091 C	T	Missense	Gcc/Acc	dppC	A129T	
B-B1	6817 G	A	Synonymous	aaG/aaA	gyrB	K565	
	7572 T	C	Missense	Tcg/Ccg	gyrA	S91P	
	21795 G	A	Missense	Ccg/Tcg	ppp	P463S	
	42967 G	C	Synonymous	ccC/ccG	mtc28	P133	
	49478 CCGTTGC	CC	Frameshift		Rv0045c		
	63771 C	T	Missense	cCg/cTg	Rv0059	P191L	
	71336 G	C	Missense	cGc/cCc	Rv0064	R906P	
	74059 C	T	Synonymous	aaG/aaA	lclD2	K151	
	79504 TCGGTGGACCCGGTGGACCCGGTGGACCCGGTGGACCCGGTGGAC	TCGGTGGACCCGGTGGACCCGGTGGACCCGGTGGACCCGGTGGAC	Codon deletion	ccggtagac/-	Rv0071	PVD22-	
	101727 G	A	Missense	gGa/gAa	ctpA	G382E	
	103756 G	T	Missense	cCc/cAc	Rv0094c	P303H	
	103896 C	G	Missense	gAg/gaC	Rv0094c	E256D	
	104944 G	A	Missense	gCc/gTc	Rv0095c	A91V	
	125830 GAAAA	GAAAAA	Frameshift		ctpI		
	131174 TGG	TGGG	Intergenic				
	154283 T	C	Missense	Tcg/Ccg	Rv0127	S18P	
	194681 G	C	Synonymous	ctC/ctG	Rv0165c	L86	
	196642 C	T	Synonymous	aaC/aaT	fadD5	N550	
	208287 A	C	Missense	gAa/gCa	Rv0176	E279A	
	223942 T	C	Missense	Tcg/Ccg	Rv0192	S127P	
	234496 C	CGT	Frameshift		Rv0197		
	293704 CTTTT	CTTTT	Intergenic				
	316129 C	G	Missense	gGc/gCc	Rv0264c	G96A	
	333892 G	C	Missense	Cgc/Ggc	PE_PGRS3	R807G	
	337820 G	A	Synonymous	ggC/ggT	PE_PGRS4	G418	
	338876 G	A	Synonymous	agC/agT	PE_PGRS4	S66	
	338960 T	C	Synonymous	gcA/gcG	PE_PGRS4	A38	
	338963 T	C	Synonymous	acA/acG	PE_PGRS4	T37	
	338984 C	T	Synonymous	gcG/gcA	PE_PGRS4	A30	
	340132 G	A	Missense	Gaa/Aaa	PPE3	E257K	
	373282 TAA	TA	Frameshift		PPE6		
	411118 CCGGGCGCG	CCGCG	Frameshift		iniA		
	424320 TCC	TCCC	Frameshift		PPE7		
	427310 TTGCGGAGTTTGCACTGCCGAGTTTGCACTGCCGAGGTTTGCACTGCC	TTGCGGAGTTTGCACTGCCGAGGTTTGCACTGCCGAGGTTTGCACTGCC	Codon deletion	ggcagtgcaaacct/-	PPE8	GSANL2436-	
	467497 CGGG	CGGGG	Frameshift		PPE9		
	467526 C	G	Missense	gGg/gCg	PPE9	G159A	
	467546 G	C	Missense	gaC/gaG	PPE9	D152E	
	467557 A	C	Missense	Ttg/Gtg	PPE9	L149V	
	467564 A	C	Missense	caT/caG	PPE9	H146Q	
	467590 T	C	Missense	Acg/Gcg	PPE9	T138A	
	467621 T	G	Synonymous	ggA/ggC	PPE9	G127	
	467638 G	T	Missense	Cag/Aag	PPE9	Q122K	
	475977 C	T	Synonymous	gcC/gcT	Rv0397	A54	
	489935 G	C	Missense	cGg/cGc	pkS6	R1402P	
	510831 TCCGGGGGCGCACCGGGG	TCCGGGGG	Codon deletion	tccccggtagcccc/tcc	ctpH	SPGAP1491S	
	580773 GGGGGACACCCGCTTGCGGGGAG	GG	Intergenic				
	583956 G	C	Synonymous	ggC/ggG	Rv0493c	G245	
	589536 G	A	Synonymous	ctG/ctA	Rv0499	L118	
	608037 A	C	Missense	cAc/cCc	Rv0515	H496P	
	623472 A	G	Missense	gAc/gCc	PE_PGRS6	D227G	
	632330 G	T	Synonymous	cgG/cgT	Rv0539	R196	
	637319 G	A	Missense	Cct/Tct	pItA	P495	
	637922 C	T	Synonymous	caG/caA	Rv0546c	Q16	
	664249 C	T	Synonymous	caG/caA	Rv0571c	Q190	
	669398 T	C	Synonymous	caA/caG	Rv0575c	Q116	
	672491 C	G	Synonymous	ggG/ggC	PE_PGRS7	G1142	
	673238 A	G	Synonymous	caT/caC	PE_PGRS7	H893	
	685461 C	G	Synonymous	gcC/gcG	yrbE2A	A111	
	685608 T	C	Synonymous	ctT/ctC	yrbE2A	L160	
	686972 T	C	Missense	tTc/tCc	mce2A	F515	
	709226 G	A	Missense	aCc/aTc	Rv0613c	T971	
	761155 C	T	Missense	tCg/tTg	rpoB	S450L	
	766823 A	C	Missense	Aag/Cag	rpoC	K1152Q	
	769962 GCCC	GCC	Frameshift		end		
	773809 G	A	Synonymous	gaG/gaA	echA4	E229	
	781922 A	G	Synonymous	aaA/aaG	rpsL	K121	
	836272 A	G	Missense	gAg/gGg	PE_PGRS9	E191G	
	836291 A	G	Synonymous	ggA/ggg	PE_PGRS9	G197	
	836658 A	G	Missense	Acc/Gcc	PE_PGRS9	T320A	
	839334 A	G	Missense	aAa/aGg	PE_PGRS10	K295R	
	839348 A	G	Missense	Agc/Ggc	PE_PGRS10	S300G	
	839949 C	G	Missense	gCb/gGg	PE_PGRS10	A500G	
	853579 C	G	Missense	gCc/gGc	phoR	A395G	
	854252 GCCCCCCC	GCCCCCCC	Intergenic				
	859131 C	CA	Frameshift		cyp123		
	859383 C	T	Missense	atG/atA	cyp123	M230I	
	874835 CC	CCGC	Frameshift		ptrBa		
	889014 AAGGAGG	A	Intergenic				
	921813 C	G	Missense	gCa/gGa	Rv0829	A80G	
	927385 A	G	Synonymous	ggA/ggG	PE_PGRS13	G675	
	947429 T	A	Missense	Tcg/Acg	Rv0850	S40T	
	947430 C	A	Nonsense	tCg/tAg	Rv0850	S40*	
	955524 A	G	Missense	Agc/Ggc	fadA	S150G	
	968426 AGCCGGGTTGGCCGG	AGCCGGGTTGGCCGGTGGCCGG	Codon insertion	-/CCGGCCAAC	PE_PGRS15	-602PAN	
	976896 TTG	T	Frameshift		PPE13		
	986463 G	C	Intergenic				
	990001 G	C	Missense	Ccc/Gcc	Rv0890c	P866A	
	993346 A	C	Missense	gTt/gGt	Rv0891c	V37G	

Table S7.3 (cont)

1010204 CGG	CGGG	Frameshift		Rv0907	
1041129 C	T	Intergenic			
1061676 GTGCTG	GTG	Intergenic			
1074558 G	A	Missense	cCc/cTc	lprP	P186L
1079927 C	A	Synonymous	acC/aCA	ctpV	T417
1093406 A	G	Synonymous	gtT/gtC	PE_PGRS17	V317
1093886 C	A	Synonymous	ggG/ggT	PE_PGRS17	G157
1093928 G	A	Synonymous	aaC/aaT	PE_PGRS17	N143
1096470 G	C	Intergenic			
1103249 C	T	Synonymous	gCc/gcT	Rv0987	A236
1107917 G	T	Missense	caC/caA	Rv0990c	H61Q
1150321 TGCCCGACAGCAAGGCC	TGCC	Frameshift		kdpD	
1159161 GCCCACC	GCC	Frameshift		Rv1034c	
1163134 T	C	Synonymous	ggA/ggG	PE8	G81
1165521 TA	TAA	Intergenic			
1168715 CTT	CTTT	Frameshift		Rv1046c	
1199547 G	A	Missense	cCa/cTa	Rv1075c	P275L
1212076 G	A	Missense	Ggc/Agc	PE_PGRS21	G173S
1218737 G	T	Missense	Ggc/Tgc	PE_PGRS22	G757C
1224367 T	C	Intergenic			
1244700 T	C	Synonymous	Tta/Cta	zwf1	L332
1276321 T	G	Missense	caA/caC	Rv1148c	Q476H
1276322 T	G	Missense	cAa/cCa	Rv1148c	Q476P
1277869 GT	GTT	Intergenic			
1307598 C	G	Missense	tGc/tCc	fadH	C210S
1313337 A	AG	Intergenic			
1328687 G	C	Missense	Ccc/Gcc	Rv1186c	P207A
1365837 CGGGG	CGGGGG	Intergenic			
1373170 G	C	Missense	cCg/cGg	Rv1230c	P343R
1374936 CAA	CA	Frameshift		Rv1232c	
1404169 T	G	Missense	aaA/aaC	cyp130	K145N
1423805 G	C	Synonymous	acC/acG	Rv1273c	T82
1440469 C	G	Synonymous	ccC/ccG	cysN	P521
1447774 C	T	Synonymous	Ctg/Ttg	argS	L466
1452071 C	A	Synonymous	ggC/ggA	thrB	G25
1457144 C	T	Missense	Cgc/Tgc	hemK	R194C
1484708 A	C	Missense	Agc/Cgc	Rv1321	S144R
1533241 G	C	Synonymous	acC/acG	PPE19	T131
1533583 G	A	Synonymous	taC/taT	PPE19	Y17
1547125 T	C	Missense	Aca/Gca	Rv1374c	T136A
1563717 C	T	Synonymous	gtC/gtT	miHf	V8
1586249 C	T	Synonymous	caG/caA	Rv1410c	Q506
1588456 A	G	Synonymous	cgT/cgC	lprG	R9
1625347 CGGG	CGGGG	Frameshift		opcA	
1631066 GGCCGCCGGTGCCGCCGGTGCCGCCGGTGCCGGTGCC	GGCCGCCGGTGCCGCCGGTGCCGCCGGTGCCGGTGCCGGTGCC	Codon deletion	ggcaccggc/-	PE_PGRS27	GTG1175-
1636826 C	A	Synonymous	ggG/ggT	PE_PGRS28	G468
1638211 T	C	Missense	Acc/Gcc	PE_PGRS28	T7A
1638212 T	C	Synonymous	gtA/gtG	PE_PGRS28	V6
1639594 C	A	Missense	cCa/cAa	Rv1453	P405Q
1645802 T	C	Missense	Aaa/Gaa	Rv1459c	K113E
1656646 TAA	TAAA	Frameshift		PE_PGRS29	
1692141 A	C	Synonymous	atA/atC	Rv1501	I84
1719322 G	A	Synonymous	ctG/ctA	Rv1524	L199
1728837 A	G	Intergenic			
1753519 GCCCC	GCCCCC	Frameshift		fadD11.1	
1759252 G	T	Synonymous	tcG/tcT	frdA	S524
1789650 C	T	Missense	Gcc/Acc	Rv1588c	A63T
1789654 A	G	Synonymous	ctT/ctC	Rv1588c	L61
1789671 C	T	Missense	Ggc/Agc	Rv1588c	A56T
1798355 G	A	Missense	gGt/gAt	Rv1597	G21D
1804409 C	A	Missense	cCa/cAa	impA	P124Q
1863700 G	C	Synonymous	ggC/ggG	PE_PGRS30	G561
1894300 GG	GGTCTTGCCGCG	Frameshift		Rv1668c	
1918690 TTGT	TTGTGT	Frameshift		tlyA	
1933988 G	A	Intergenic			
1944402 T	C	Missense	gTc/gCc	Rv1716	V276A
1944642 CT	C	Frameshift		Rv1717	
1967237 C	A	Missense	cGa/ctA	Rv1739c	R134L
1983313 T	G	Missense	aAc/aCc	PPE24	N488T
1986623 GTT	GTTT	Frameshift		Rv1754c	
2045310 A	G	Synonymous	atT/atC	PE_PGRS32	I511
2052035 G	T	Missense	Gtg/Ttg	PPE33	V252L
2055271 A	G	Missense	ctA/cCa	Rv1812c	L30P
2058257 T	G	Start lost	tTg/tGg	Rv1816	L1W
2074565 C	G	Intergenic			
2075094 C	T	Missense	gCb/gTg	Rv1830	A85V
2094911 ACAGCGTCAGCGTCAG	ACAGCGTCAG	Codon deletion	actgacgt/act	gnd1	TDA88T
2096186 A	G	Synonymous	acT/acC	Rv1846c	T138
2109523 CGG	CGGG	Intergenic			
2137521 AC	ACTCCGATCACC	Frameshift		Rv1888c	
2143328 G	C	Missense	Gtg/Ctg	Rv1895	V270L
2197148 C	A	Missense	aCc/aAc	Rv1945	T387N
2207525 C	T	Intergenic			
2207591 T	TC	Intergenic			
2211826 A	G	Synonymous	aaA/aaG	mce3C	K67
2231879 T	C	Missense	gTc/gCc	Rv1988	V67A
2268725 A	AG	Frameshift		Rv2024c	
2269780 T	C	Missense	gAc/gCc	Rv2024c	D154G
2278442 C	G	Synonymous	cgG/cgC	Rv2030c	R15
2300205 A	AC	Frameshift		pkS12	
2300555 A	G	Synonymous	gaT/gaC	pkS12	D2144
2335075 A	G	Missense	gAa/gGa	Rv2078	EGG
2340621 C	G	Missense	cCc/cGc	Rv2082	P638R
2345037 C	A	Synonymous	ctC/ctA	pknJ	L209
2348446 C	G	Missense	tTc/tTg	Rv2090	F358L
2357268 TGCCGCCGCCGCCGCCG	TGCCGCCGCCGCCGCCG	Intergenic			
2365413 AT	ATT	Intergenic			
2368564 TAA	TA	Intergenic			
2382085 AGTG	AG	Frameshift		PPE37	
2415656 G	C	Missense	Cgg/Ggg	murD	R250G
2424925 A	G	Intergenic			
2434782 A	G	Intergenic			
2439204 A	G	Intergenic			
2439401 A	G	Missense	Tac/Cac	Rv2177c	Y183H
2468058 C	T	Intergenic			
2470886 AAACCTAGCCGCGA	AA	Codon change and Codon deletion	aaacttagccgcgag/aag	Rv2206	KLSRE89K
2499726 G	A	Missense	Gat/Aat	Rv2226	D299N
2503625 C	T	Synonymous	ctG/ctA	Rv2230c	L328
2509140 G	C	Missense	tCc/tGc	cobD	S79C
2523205 GCG	GCGCCG	Intergenic			
2534562 GGAG	GG	Frameshift		Rv2262c	
2573756 C	A	Intergenic			
2617632 A	AT	Intergenic			
2626018 T	C	Missense	gAg/gGg	esxO	E52G
2626026 A	G	Synonymous	gcT/gcC	esxO	A49
2626095 C	G	Synonymous	gcG/gcC	esxO	A26
2626155 C	T	Synonymous	caG/caA	esxO	Q6
2654371 G	A	Synonymous	aaC/aaT	hrcA	N241
2695378 C	G	Missense	gGc/gCc	cysW	G141A
2704884 AC	ACAGCGACCATATCGCCGAGCTC	Codon insertion	-/AGCGACCATATCGCCGAGCTC	Rv2407	-645SDHIAEL
2715342 C	T	Intergenic			

Table S7.3 (cont)

2734074 T	C	Missense	gTc/gCc	rbsK	V282A	
2830525 C	A	Missense	aCg/aAg	Rv2513	T122K	
2865760 A	G	Missense	Acc/Gcc	Rv2542	T211A	
2868659 C	G	Synonymous	gCc/gcG	Rv2547	A18	
2881597 AGGGGGG	AGGGGGG	Frameshift		Rv2561		
2945167 G	T	Intergenic				
2955571 A	G	Intergenic				
3011401 A	G	Stop lost	Tga/Cga	Rv2694c	*123R	
3028101 ACC	ACCC	Frameshift		Rv2715		
3054081 A	G	Synonymous	ggA/ggG	PE_PGRS47	G56	
3073868 T	C	Missense	Acc/Gcc	thyA	T202A	
3077039 C	A	Missense	gGc/gTc	PPE43	G347V	
3080795 A	G	Missense	cTc/cCc	Rv2771c	L80P	
3131469 TTGT	TTGTGCGCGATGT	Codon insertion	-/ACATCGCGCG	Rv2823c	-101TSP	
3133536 T	C	Missense	Aaa/Gaa	Rv2825c	K2E	
3158935 G	C	Missense	Cgc/Ggc	Rv2850c	R374G	
3178445 C	G	Intergenic				
3179242 ACGGCGGCGCGG	ACGGCGGCGG	Codon deletion	gacgcc/gac	Rv2867c	DA20D	
3190145 TCCCCC	TCCCCC	Frameshift		Rv2880c		
3191358 G	A	Synonymous	atC/atT	cdsA	I88	
3226181 A	C	Synonymous	cgt/cgG	ffh	R35	
3232815 A	G	Intergenic				
3247853 C	T	Synonymous	gCc/gcT	ppsA	A803	
3247856 G	C	Synonymous	cGg/cgC	ppsA	R804	
3247874 G	A	Synonymous	cGg/cgA	ppsA	R810	
3247883 T	C	Synonymous	agT/agC	ppsA	S813	
3302324 AGCGAG	A	Frameshift		fadD29		
3312632 C	T	Nonsense	tgG/tgA	Rv2959c	W69*	
3354896 C	T	Synonymous	gaG/gaA	serA1	E58	
3363338 A	G	Intergenic				
3377271 G	C	Missense	Ctg/Gtg	PPE46	L325V	
3377275 G	C	Synonymous	gCc/gcG	PPE46	A323	
3377281 T	C	Synonymous	ggA/ggG	PPE46	G321	
3377305 T	C	Synonymous	gtA/gtG	PPE46	V313	
3377347 C	A	Synonymous	ggG/ggT	PPE46	G299	
3379718 T	C	Synonymous	ggA/ggG	PPE47	G245	
3379742 T	C	Synonymous	gtA/gtG	PPE47	V237	
3379751 A	C	Synonymous	gtT/gtG	PPE47	V234	
3379784 C	A	Synonymous	ggG/ggT	PPE47	G223	
3402816 C	T	Missense	Gga/gAa	serB2	G116E	
3415180 ACACCTAGGGGGTGGCACCTAGGGGGTGGCAC	ACACCTAGGGGGTGGCAC	Intergenic				
3418328 T	G	Missense	gAt/gcT	Rv3057c	D112A	
3418330 G	A	Synonymous	caC/caT	Rv3057c	H111	
3473996 GA	GAA	Intergenic				
3480375 GAAA	GAA	Frameshift		Rv3113		
3484012 T	G	Synonymous	gcT/gcG	cysA3	A13	
3503895 C	T	Missense	cGg/cTg	Rv3137	P168L	
3504930 C	T	Synonymous	Ctg/Ttg	pflA	L246	
3510642 T	C	Missense	Agc/Ggc	PPE52	S226G	
3519294 GGCC	GGCCGCC	Codon change and Codon insertion	cac/cGCCac	nuoH	H6RH	
3554787 G	A	Missense	gGa/gAa	Rv3189	G49E	
3561155 G	A	Missense	gCc/gTc	Rv3193c	A673V	
3580636 CTTT	CTT	Frameshift		lipV		
3590686 GT	GCT	Intergenic				
3604821 G	C	Synonymous	gCc/gcC	Rv3228	A32	
3661530 C	T	Synonymous	caG/caA	birA	Q161	
3689523 G	T	Nonsense	tgC/tgA	lpdA	C472*	
3730741 G	A	Synonymous	ggC/ggT	PPE54	G2065	
3736261 A	G	Synonymous	ggT/ggC	PPE54	G225	
3739913 G	A	Synonymous	acC/acT	PE_PGRS50	T954	
3769550 G	A	Synonymous	taC/taT	Rv3355c	Y86	
3779671 CGGCAACGGTGGAACGGTGGCAACGGTGGAACGGTG	CGGCAACGGTGGAACGGTGGCAACGGTGGCAACGGTG	Codon change and Codon insertion	gcc/gGCAACGGTGcc	PE_PGRS51	A378NGNA	
3794884 G	A	Intergenic				
3840393 A	G	Missense	aTg/aCg	alr	M343T	
3847378 G	C	Missense	Gac/Cac	PPE59	D72H	
3847380 C	A	Missense	gAc/gaA	PPE59	D72E	
3859376 C	T	Missense	gGg/gAg	Rv3439c	G96E	
3862472 GAA	GA	Intergenic				
3883707 T	C	Synonymous	ctT/ctC	Rv3466	L61	
3883711 G	A	Missense	Gcc/Acc	Rv3466	A63T	
3884906 A	G	Missense	Aaa/Gaa	Rv3467	K315E	
3890778 CTGA	C	Intergenic				
3892671 A	G	Synonymous	gtT/gtC	kgfP	V350	
3930476 ACGGCG	ACGGCGGACCGGCGG	Codon change and Codon insertion	gtt/gGCACCGCGCgtt	PE_PGRS53	V1305GTGV	
3934699 G	A	Missense	aGc/aAc	PE_PGRS54	S1232N	
3940802 A	G	Missense	Aac/Gac	PE_PGRS55	N396D	
3942481 C	G	Missense	gCa/ggG	PE_PGRS56	A253G	
3942640 T	C	Missense	aTt/aCt	PE_PGRS56	I306T	
3980059 AGGCGGCGGCGCGC	AGGCGGCGGC	Codon change and Codon deletion	ggccgc/gcc	PPE64	AA30A	
4037283 T	G	Synonymous	ggA/ggC	PE_PGRS59	G256	
4038287 G	A	Synonymous	aaC/aaT	clpC1	N806	
4093879 TGGGGG	TGGGG	Frameshift		PE_PGRS60		
4095001 CG	C	Frameshift		Rv3655c		
4120983 A	G	Intergenic				
4135112 G	A	Missense	atG/atA	Rv3693	M129I	
4187817 A	G	Missense	gAt/gGt	Rv3737	D40G	
4197138 CTT	CTTT	Intergenic				
4198611 CGGGGG	CGGGG	Intergenic				
4218350 T	C	Missense	aAg/aGg	Rv3773c	K159R	
4247702 C	A	Missense	CgG/AcG	embB	P397T	
4264219 T	G	Synonymous	ggA/ggC	Rv3802c	G49	
4287370 A	G	Missense	cAg/cGg	Rv3822	Q217R	
4293072 G	A	Missense	Ctt/Ttt	papA1	L35F	
4320050 A	G	Intergenic				
4336090 A	AT	Intergenic				
4338595 GC	G	Intergenic				
4338732 G	A	Intergenic				
4353201 CCCGCCGCCGCC	CCGCCGCC	Codon change and Codon deletion	ccgcc/cca	Rv3876	PP67P	
4373475 C	G	Synonymous	gtG/gtC	Rv3889c	V52	
4373496 C	G	Synonymous	gtG/gtC	Rv3889c	V45	
4382275 G	T	Missense	Caa/Aaa	Rv3896c	Q193K	
4383144 CC	CCGGGGC	Frameshift		Rv3897c		
4385177 G	C	Missense	cCa/cGa	Rv3899c	P68R	
4395964 C	A	Synonymous	acC/acA	Rv3909	T591	
4400660 ACCC	ACC	Frameshift		sigM		
4410200 G	A	Synonymous	ctC/ctT	Rv3922c	L72	
B-C	1567562 C	T	Synonymous	aaC/aaT	metK	N246
	3379757 A	C	Synonymous	gCt/gcG	PPE47	A232
D-C	2998574 G	T	Synonymous	ggC/ggA	dxs1	G465
	3261702 G	A	Synonymous	gCg/gcA	ppsC	A2006
D-D1	42967 G	C	Synonymous	ccC/ccG	mtc28	P133
	63771 C	T	Missense	cGg/cTg	Rv0059	P191L
	71336 G	C	Missense	cGc/cCc	Rv0064	R906P
	71585 CG	CGAG	Intergenic			
	103756 G	T	Missense	cCc/cAc	Rv0094c	P303H

Table S7.3 (cont)

194681 G	C	Synonymous	ctC/ctG	Rv0165c	L86
208287 A	C	Missense	gAa/gCa	Rv0176	E279A
467557 A	C	Missense	Ttg/Gtg	PPE9	L149V
467564 A	C	Missense	caT/caG	PPE9	H146Q
467621 T	G	Synonymous	ggA/ggC	PPE9	G127
467638 G	T	Missense	CaG/AaG	PPE9	Q122K
475977 C	T	Synonymous	gcC/gcT	Rv0397	A54
583956 G	C	Synonymous	ggC/ggG	Rv0493c	G245
632330 G	T	Synonymous	cgG/cgT	Rv0539	R196
637922 C	T	Synonymous	caG/caA	Rv0546c	Q16
669398 T	C	Synonymous	caA/caG	Rv0575c	Q116
673238 A	G	Synonymous	caT/caC	PE_PGRS7	H893
709226 G	A	Missense	aCc/aTc	Rv0613c	T971
761110 A	T	Missense	gAc/gTc	rpoB	D435V
773809 G	A	Synonymous	gaG/gaA	echA4	E229
836538 A	G	Missense	Aac/Gac	PE_PGRS9	N280D
836658 A	G	Missense	Acc/Gcc	PE_PGRS9	T320A
853579 C	G	Missense	gCc/gCc	phoR	A395G
859383 C	T	Missense	atG/atA	cyp123	M230I
921813 C	G	Missense	gCa/gGa	Rv0829	A80G
947430 C	A	Nonsense	tGg/tAg	Rv0850	S40*
1041129 C	T	Intergenic			
1074558 G	A	Missense	cCc/cTc	lprP	P186L
1103249 C	T	Synonymous	gcC/gcT	Rv0987	A236
1218737 G	T	Missense	Ggc/Tgc	PE_PGRS22	G757C
1307598 C	G	Missense	tGc/tCc	fadH	C210S
1447774 C	T	Synonymous	Ctg/Ttg	argS	L466
1457144 C	T	Missense	Cgc/Tgc	hemK	R194C
1484708 A	C	Missense	Agc/Cgc	Rv1321	S144R
1588456 A	G	Synonymous	cgT/cgC	lprG	R9
1692141 A	C	Synonymous	atA/atC	Rv1501	I84
1697055 A	G	Missense	cTa/cCa	Rv1507c	L123P
1719322 G	A	Synonymous	ctG/ctA	Rv1524	L199
1789671 C	T	Missense	Gcg/Acg	Rv1588c	A56T
1798355 G	A	Missense	gGt/gAt	Rv1597	G21D
1944402 T	C	Missense	gTc/gCc	Rv1716	V276A
1986625 TCATC	TTGCATC	Frameshift		Rv1754c	
2045310 A	G	Synonymous	atT/atC	PE_PGRS32	IS11
2052035 G	T	Missense	Gtg/Ttg	PPE33	V252L
2055271 A	G	Missense	cTa/cCa	Rv1812c	L30P
2058257 T	G	Start lost	tTg/tGg	Rv1816	L1W
2143328 G	C	Missense	Gtg/Ctg	Rv1895	V270L
2269780 T	C	Missense	gAc/gCc	Rv2024c	D154G
2278442 C	G	Synonymous	cgG/cgC	Rv2030c	R15
2300205 ATGT	ACTGT	Frameshift		pkS12	
2348446 C	G	Missense	tTc/ttG	Rv2090	F358L
2401883 C	T	Intergenic			
2509140 G	C	Missense	tCc/tGc	cobD	S79C
2626018 T	C	Missense	gAg/gGg	esxO	E52G
2748308 A	G	Missense	Tcc/Ccc	valS	S640P
2811885 G	A	Missense	gCb/gTg	pdhA	A71V
2868659 C	G	Synonymous	gcC/gcG	Rv2547	A18
2955571 A	G	Intergenic			
3073868 T	C	Missense	Acc/Gcc	thyA	T202A
3077039 C	A	Missense	gCc/gTc	PPE43	G347V
3080795 A	G	Missense	cTc/cCc	Rv2771c	L80P
3247856 G	C	Synonymous	cgG/cgC	ppsA	R804
3247883 T	T	Synonymous	agT/agC	ppsA	S813
3354896 C	C	Synonymous	gaG/gaA	serA1	E58
3377305 T	C	Synonymous	gtA/gtG	PPE46	V313
3379718 T	C	Synonymous	ggA/ggG	PPE47	G245
3379742 T	C	Synonymous	gtA/gtG	PPE47	V237
3379751 A	C	Synonymous	gtT/gtG	PPE47	V234
3379763 G	A	Synonymous	gcC/gcT	PPE47	A230
3484012 T	G	Synonymous	gcT/gcG	cysA3	A13
3503895 C	T	Missense	cGc/cTg	Rv3137	P168L
3510642 T	C	Missense	Agc/Ggc	PPE52	S226G
3561155 G	A	Missense	gCc/gTc	Rv3193c	A673V
3604821 G	C	Synonymous	gcG/gcC	Rv3228	A32
3892671 A	G	Synonymous	gtT/gtC	kgpP	V350
3934542 T	G	Missense	Tct/Gct	PE_PGRS54	S1180A
3942640 T	C	Missense	aTt/aCt	PE_PGRS56	I306T
4187817 A	G	Missense	gAt/gGt	Rv3737	D40G
4218350 T	C	Missense	aAg/aGg	Rv3773c	K159R
4287370 A	G	Missense	cAg/cGg	Rv3822	Q217R
4373496 C	G	Synonymous	gtG/gtC	Rv3889c	V45
4385177 G	C	Missense	cCa/cGa	Rv3899c	P68R
4395964 C	A	Synonymous	acC/acA	Rv3909	T591
D-E					
2378 G	A	Synonymous	ttG/ttA	dnaN	L109
6817 G	T	Synonymous	aaG/aaA	gyrB	K565
196642 C	T	Synonymous	aaC/aaT	fadO5	N550
223942 T	C	Missense	Tcg/Ccg	Rv0192	S127P
589536 G	A	Synonymous	ctG/ctA	Rv0499	L118
608037 A	C	Missense	cAc/cCc	Rv0515	H496P
685608 T	C	Synonymous	ctT/ctC	yrbE2A	L160
761155 C	T	Missense	tGg/tTg	rpoB	S450L
947429 T	A	Missense	Tcg/Acg	Rv0850	S40T
955524 A	G	Missense	Agc/Ggc	fadA	S150G
1079927 C	A	Synonymous	acC/acA	ctpV	T417
1093406 A	G	Synonymous	gtT/gtC	PE_PGRS17	V317
1107917 G	T	Missense	caC/caA	Rv0990c	H61Q
1199547 G	A	Missense	cCa/cTa	Rv1075c	P275L
1533583 G	A	Synonymous	taC/taT	PPE19	Y17
1547125 T	C	Missense	Aca/Gca	Rv1374c	T136A
1645802 T	C	Missense	Aaa/Gaa	Rv1459c	K113E
1689562 A	G	Missense	tTc/tCc	Rv1498c	F120S
1759252 G	T	Synonymous	tcG/tcT	frdA	S524
1789650 C	T	Missense	Gcc/Acc	Rv1588c	A63T
1789654 A	G	Synonymous	ctT/ctC	Rv1588c	L61
1967237 C	A	Missense	cGa/ctA	Rv1739c	R134L
1983313 T	G	Missense	aAc/aCc	PPE24	N488T
2096186 A	G	Synonymous	acT/acC	Rv1846c	T138
2207525 C	T	Intergenic			
2231879 T	C	Missense	gTc/gCc	Rv1988	V67A
2335075 A	G	Missense	gAa/gGa	Rv2078	E6G
2345037 C	A	Synonymous	ctC/ctA	pknJ	L209
2468058 C	T	Intergenic			
2499726 G	A	Missense	Gat/Aat	Rv2226	D299N
2503625 C	T	Synonymous	ctG/ctA	Rv2230c	L328
2654371 G	A	Synonymous	aaC/aaT	hrcA	N241
2695378 C	G	Missense	gGc/gCc	cysW	G141A
2830525 C	A	Missense	aCb/aAg	Rv2513	T122K
2962129 C	A	Missense	Gta/Tta	PE_PGRS46	V105L
3377271 G	C	Missense	Ctg/Gtg	PPE46	L325V
3377275 G	C	Synonymous	gcC/gcG	PPE46	A323
3379712 G	C	Synonymous	gcC/gcG	PPE47	A247
3504930 C	T	Synonymous	Ctg/Ttg	pflA	L246
3654567 G	A	Missense	Gcg/Acg	Rv3272	A374T
3661530 C	T	Synonymous	caG/caA	birA	Q161
3689523 G	T	Nonsense	tgC/tgA	lpdA	C472*

Table S7.3 (cont)

3794884 G		A	Intergenic					
3859376 C		T	Missense					Rv3439c G96E
4038287 G		A	Synonymous	gGg/gAg				clpC1 N806
4135112 G		A	Missense	aaC/aaT				Rv3693 M129H
4338732 G		A	Intergenic	atG/atA				
4410200 G		A	Synonymous	ctC/ctT			Rv3922c	L72
E-E1								
7582 A		G	Missense	gAc/gGc			gyrA	D94G
21795 G		A	Missense	CcG/TpG			ppp	P463S
42967 G		C	Synonymous	ccC/ccG			mtc28	P133
63771 C		T	Missense	cCg/cTg			Rv0059	P191L
71336 G		C	Missense	cGc/cCc			Rv0064	R906P
74059 C		T	Synonymous	aaG/aaA			icd2	K151
101727 G		A	Missense	gGA/gAA			ctpA	G382E
103756 G		T	Missense	cCc/cAc			Rv0094c	P303H
104944 G		A	Missense	gCc/gTc			Rv0095c	A91V
154283 T		C	Missense	TcG/CcG			Rv0127	S18P
179886 G		A	Synonymous	gAc/gpT			PE2	D337
194681 G		C	Synonymous	ctC/ctG			Rv0165c	L86
208287 A		C	Missense	gAA/gCa			Rv0176	E279A
316129 C		G	Missense	gGc/gCc			Rv0264c	G96A
333892 G		C	Missense	Cgc/Ggc			PE_PGRS3	R807G
337820 G		A	Synonymous	ggC/ggT			PE_PGRS4	G418
338020 A		C	Missense	TgC/Ggc			PE_PGRS4	C352G
338100 T		C	Missense	aAc/AGc			PE_PGRS4	N325S
338876 G		A	Synonymous	agC/agT			PE_PGRS4	S66
338960 T		C	Synonymous	gcA/gcG			PE_PGRS4	A38
340132 G		A	Missense	Gaa/GAa			PPE3	E257K
467526 C		G	Missense	gGg/gCc			PPE9	G159A
467546 G		C	Missense	gaC/gaG			PPE9	D152E
467557 A		C	Missense	Ttg/Gtg			PPE9	L149V
467564 A		C	Missense	caT/caG			PPE9	H146Q
467590 T		C	Missense	Acg/Gcg			PPE9	T138A
467621 T		G	Synonymous	ggA/ggC			PPE9	G127
467638 G		T	Missense	Cag/Aag			PPE9	Q122K
475977 C		T	Synonymous	gcC/gcT			Rv0397	AS4
489935 G		C	Missense	cGg/cCg			pkx6	R1402P
490949 C		G	Missense	tCc/tGc			fgd1	S56C
583956 G		C	Synonymous	ggC/gggG			Rv0493c	G24S
623472 A		G	Missense	gAc/gGc			PE_PGRS6	D227G
623508 C		G	Missense	gCc/gGc			PE_PGRS6	A239G
623230 G		T	Synonymous	cGg/cgT			Rv0539	R196
637319 G		A	Missense	Cct/Tct			pilA	P49S
637922 C		T	Synonymous	caG/caA			Rv0546c	Q16
664249 C		T	Synonymous	caG/caA			Rv0571c	Q190
669398 T		C	Synonymous	caA/cag			Rv0575c	Q116
672491 C		G	Synonymous	ggG/gggC			PE_PGRS7	G1142
673238 A		G	Synonymous	caT/caC			PE_PGRS7	H893
685461 C		G,T	Synonymous	gcC/gcG			yrbE2A	A111
686972 T		C	Missense	tTc/tCc			mce2A	F51S
709226 G		A	Missense	aCc/atC			Rv0613c	T97I
773809 G		A	Synonymous	gGg/gaA			echA4	E229
781922 A		G,T	Missense	aaA/aaT			rpsL	K121N
836272 A		G,T	Missense	gAb/gGg			PE_PGRS9	E191G
836291 A		G	Synonymous	ggA/gggG			PE_PGRS9	G197
836426 A		C	Synonymous	ctA/ctC			PE_PGRS9	L242
836454 A		G	Missense	AcI/GcI			PE_PGRS9	T252A
836538 A		G	Missense	Aac/Gac			PE_PGRS9	N280D
836658 A		G	Missense	Acc/Gcc			PE_PGRS9	T320A
839334 A		G	Missense	aAa/agA			PE_PGRS10	K295R
839348 A		G	Missense	Agc/Ggc			PE_PGRS10	S300G
839949 C		G,T	Missense	gGg/gGg			PE_PGRS10	AS00G
853579 C		G	Missense	gCc/gGc			phoR	A395G
859383 C		T	Missense	atG/atA			cyp123	M230I
921813 C		G	Missense	gCa/gGa			Rv0829	A80G
927110 A		G	Missense	Agt/Ggt			PE_PGRS13	S584G
927385 A		G	Synonymous	ggA/gggG			PE_PGRS13	G67S
947430 C		A	Nonsense	tCg/tAg			Rv0850	S40*
986463 G		C	Intergenic					
990001 G		C	Missense	Ccc/Gcc			Rv0890c	P866A
993346 A		C	Missense	gTl/gGt			Rv0891c	V37G
1041129 C		T	Intergenic					
1074558 G		A	Missense	cCc/ctC			lprP	P186L
1093886 C		A	Synonymous	ggG/ggT			PE_PGRS17	G157
1103249 C		T	Synonymous	gcC/gcT			Rv0987	A236
1163134 T		C,A	Synonymous	ggA/gggG			PE8	G81
1189431 TGCCGCCGCCGCCG	TGCCGCCGCCGCCG		Codon deletion	aacggc/aac			PE_PGRS19	NG326N
1212076 G		A	Missense	Ggc/Agc			PE_PGRS21	G173S
1218737 G		T	Missense	Ggc/Tgc			PE_PGRS22	G757C
1224367 T		C	Intergenic					
1244700 T		C	Synonymous	Tta/Cta			zwf1	L332
1276321 T		G	Missense	caA/caC			Rv1148c	Q476H
1276322 T		G	Missense	cAa/cCa			Rv1148c	Q476P
1307598 C		G	Missense	tGc/tCc			fadH	C210S
1328687 G		C	Missense	Ccc/Gcc			Rv1186c	P207A
1373170 G		C,A	Missense	cGc/cGg			Rv1230c	P343R
1404169 T		G	Missense	aaA/aaC			cyp130	K145N
1423805 G		C	Synonymous	acC/acG			Rv1273c	T82
1440469 C		G	Synonymous	ccC/ccG			cysN	PS21
1447774 C		T	Synonymous	Ctg/Ttg			argS	L466
1452071 C		A	Synonymous	ggC/ggA			thrB	G25
1457144 C		T	Missense	Cgc/Tgc			hemK	R194C
1484708 A		C	Missense	Agc/Ggc			Rv1321	S144R
1563717 C		T	Synonymous	gtC/gtT			mlhF	V8
1586249 C		T	Synonymous	caG/caA			Rv1410c	Q506
1588456 A		G	Synonymous	cgt/cGc			lprG	R9
1631066 GGCGCCGGTGCCGCCGGTGCCGCCGGTGCCGGTGGCCGGTGCC	GGCGCCGGTGCCGCCGGTGCCGCCGGTGCCGGTGGCCGGTGGCCGGTGCC		Codon deletion	ggcaccggc/-			PE_PGRS27	GTG1175-
1634580 T		C	Missense	tTA/ttG			PE_PGRS27	L16
1634581 A		G	Missense	tTA/tCa			PE_PGRS27	L16S
1636826 C		A	Synonymous	ggG/ggT			PE_PGRS28	G468
1638211 T		C	Missense	Acc/Gcc			PE_PGRS28	T7A
1638212 T		C	Synonymous	gtA/gtG			PE_PGRS28	V6
1639594 C		C	Missense	cCa/cAa			Rv1453	P405Q
1692141 A		C	Synonymous	atA/atC			Rv1501	I84
1719322 G		A	Synonymous	ctG/ctA			Rv1524	L199
1728837 A		G	Intergenic					
1789671 C		T	Missense	Gcg/Agc			Rv1588c	A56T
1798355 G		A	Missense	gGT/gAt			Rv1597	G21D
1804409 C		A	Missense	cCa/cAa			impA	P124Q
1863700 G		C,A	Synonymous	ggC/gggG			PE_PGRS30	G56I
1933988 G		A	Intergenic					
1944402 T		C	Missense	gTc/gCc			Rv1716	V276A
2045310 A		G	Synonymous	atT/atC			PE_PGRS32	I511
2052035 G		T	Missense	Gtg/Ttg			PPE33	V252L
2055271 A		G	Missense	cTa/cCa			Rv1812c	L30P
2058257 T		G	Start lost	tTg/tGg			Rv1816	L1W
2074565 C		G	Intergenic					
2143328 G		C	Missense	Gtg/Ctg			Rv1895	V270L
2211826 A		G	Synonymous	aaA/aaG			mce3C	K67
2269780 T		C	Missense	gAc/gGc			Rv2024c	D154G

Table S7.3 (cont)

2278442 C	G	Synonymous	cGg/cgC	Rv2030c	R15
2288883 A	G	Missense	cTg/cCg	pncA	L120P
2300237 A	G	Synonymous	gcT/gcC	pkx12	A2250
2300552 T	G	Synonymous	ccA/ccC	pkx12	P2145
2300555 A	G	Synonymous	gaT/gaC	pkx12	D2144
2340621 C	G	Missense	cCc/cCc	Rv2082	P638R
2348446 C	G	Missense	ttC/ttG	Rv2090	F358L
2415656 G	C,A	Missense	Cgg/Ggg	murD	R250G
2424925 A	G	Intergenic			
2439204 A	G,T	Intergenic			
2509140 G	C	Missense	tCc/tGc	cobD	S79C
2573756 C	A	Intergenic			
2626018 T	C	Missense	gAg/gGg	esxO	E52G
2626026 A	G	Synonymous	gcT/gcC	esxO	A49
2626155 C	T	Synonymous	caG/caA	esxO	Q6
2715342 C	T	Intergenic			
2734074 T	C	Missense	gTc/gCc	rbsK	V282A
2784613 A	G	Intergenic			
2865760 A	G	Missense	Acc/Gcc	Rv2542	T211A
2868659 C	G	Synonymous	gcC/gcG	Rv2547	A18
2955571 A	G	Intergenic			
3054081 A	G	Synonymous	ggA/ggG	PE_PGRS47	G56
3080795 A	G	Missense	cTc/ccC	Rv2771c	L80P
3133536 T	C	Missense	Aaa/Gaa	Rv2825c	K2E
3158935 G	C	Missense	Cgc/Ggc	Rv2850c	R374G
3178445 C	G	Intergenic			
3191358 G	A	Synonymous	atC/atT	cdsA	I88
3226181 A	C	Synonymous	cgt/cgG	ffh	R35
3232815 A	G	Intergenic			
3247853 C	T	Synonymous	gcC/gcT	ppsA	A803
3247874 G	A	Synonymous	cGg/cgA	ppsA	R810
3247877 T	C	Synonymous	ttT/ttC	ppsA	F811
3247883 T	C	Synonymous	agT/agC	ppsA	S813
3312632 C	T	Nonsense	tgG/tgA	Rv2959c	W69*
3354896 C	T	Synonymous	gaG/gaA	serA1	E58
3363338 A	G	Intergenic			
3377305 T	C	Synonymous	gtA/gtG	PPE46	V313
3377347 C	A	Synonymous	ggG/ggT	PPE46	G299
3379718 T	C	Synonymous	ggA/gggG	PPE47	G245
3379751 A	C	Synonymous	gtT/gtG	PPE47	V234
3379763 G	A	Synonymous	gcC/gcT	PPE47	A230
3379784 C	A	Synonymous	ggG/ggT	PPE47	G223
3402816 C	T	Missense	gGa/gAa	serB2	G116E
3418328 T	G	Missense	gAt/gcT	Rv3057c	D112A
3418330 G	A	Synonymous	caC/caT	Rv3057c	H111
3484012 T	G	Synonymous	gcT/gcG	cysA3	A13
3503895 C	T	Missense	cGg/cTg	Rv3137	P168L
3510642 T	C	Missense	Agc/Ggc	PPE52	S226G
3554787 G	A	Missense	gGa/gAa	Rv3189	G49E
3560178 C	T	Intergenic			
3561155 G	A	Missense	gCc/gTc	Rv3193c	A673V
3604821 G	C	Synonymous	gcG/gcC	Rv3228	A32
3739913 G	A	Synonymous	acC/acT	PE_PGRS50	T954
3769550 G	A	Synonymous	taC/taT	Rv3355c	Y86
3796409 TTCA	T	Intergenic			
3841083 A	C	Missense	cTg/cGg	alr	L113R
3883707 T	C	Synonymous	ctT/ctC	Rv3466	L61
3883711 G	A	Missense	Gcc/Acc	Rv3466	A63T
3890778 CTGA	C	Intergenic			
3892671 A	G	Synonymous	gtT/gtC	kgtP	V350
3928784 CCGCGGGGCCGGCGG	CCGGCGGGGCCGGCGGTACGGCGGGCCGGCG	Codon change and Codon insertion	ggC/ggTAACGGCGGGGCCGGCGGc	PE_PGRS53	G744GNGGAGG
3934542 T	G	G	Tct/Cct	PE_PGRS54	S1180A
3934699 G	A	Missense	aGc/aAc	PE_PGRS54	S1232N
3940802 A	G	Missense	Aac/Gac	PE_PGRS55	N296D
3942481 C	G	Missense	gCb/gGg	PE_PGRS56	A253G
3942640 T	C	Missense	aTt/aCt	PE_PGRS56	I306T
3943124 C	T	Synonymous	ggC/ggT	PE_PGRS56	G467
4037283 T	G	Synonymous	ggA/gggC	PE_PGRS59	G256
4187817 A	G	Missense	gAt/gcT	Rv3737	D40G
4218350 T	C	Missense	aAg/aGg	Rv3773c	K159R
4247429 A	G	Missense	Atg/Gtg	embB	M306V
4287370 A	G	Missense	cAg/cGg	Rv3822	Q217R
4293072 G	A	Missense	Ctt/Ttt	papA1	L35F
4320050 A	G	Intergenic			
4373475 C	G	Synonymous	gtG/gtC	Rv3889c	V52
4373496 C	T	Synonymous	gtG/gtC	Rv3889c	V45
4382275 G	G	Missense	Caa/Aaa	Rv3896c	Q193K
4385177 G	C	Missense	cCa/cGa	Rv3899c	P68R
4395964 C	A	Synonymous	acC/acA	Rv3909	T591
E-F					
49478 CCGTTGC	CC	Frameshift		Rv0045c	
79504 TCGGTGGACCCGGTGGACCCGGTGG	TCGGTGGACCCGGTGGACCCGGTGGTGG	Codon deletion	ccggtggac/-	Rv0071	PVD22-
125830 GAAAA	ACCCGGTGGAC			ctpl	
131174 TGG	TGGG	Frameshift			
234496 C	CGT	Frameshift		Rv0197	
293704 CTTTT	CTTTT	Intergenic			
373282 TAA	TA	Frameshift		PPE6	
411118 CCGGGCGCG	CCGCG	Frameshift		inia	
424320 TCC	TCCC	Frameshift		PPE7	
427310 TTGCCGAGTTTGCACTGCCGAGTTTGCACTGCC	TTGCCGAGTTTGCACTGCCGAGTTTGCACTGCC	Codon deletion	ggcagtgcaaacct/-	PPE8	GSANL2436-
510831 TCCGGGGGCGCACCAGGGGG	GAGGTTTGCACTGCCGAGTTTGCACTGCC				
580773 GGGGGCACCACCGCTTTCGGGGGAG	GAGGTTTGCACTGCC	Codon deletion	tccccgggtgcgcc/tcc	ctpH	SPGAP14915
769962 GCCC	GG				
859131 C	GCC	Frameshift		end	
874835 CC	CA	Frameshift		cyp123	
889014 AAGGAGG	CCGC	Frameshift		ptrBa	
968426 AGCGGGTTGGCCGG	A	Intergenic			
976896 TTG	AGCCGGTTGGCCGGTTGGCCGG	Codon insertion	-/CCGCCCAAC	PE_PGRS15	-602PAN
1010204 CGG	T	Frameshift		PPE13	
1150321 TGCCGACGCAAGGCC	CGGG	Frameshift		Rv0907	
1159161 GCCCACC	TGCC	Frameshift		kdpD	
1165521 TA	GCC	Frameshift		Rv1034c	
1168715 CTT	TAA	Intergenic			
1277869 GT	CTTT	Frameshift		Rv1046c	
1313337 A	GTT	Intergenic			
1365837 CGGG	AG	Intergenic			
1374936 CAA	CGGGG	Intergenic			
1527449 GT	CA	Frameshift		Rv1232c	
1625347 CGGG	GTT	Frameshift		Rv1358	
1753519 GCCCC	CGGGG	Frameshift		opcA	
1894300 GGT	GCCCC	Frameshift		fadD11.1	
1986623 GTT	GGTCTTGCCGCG	Frameshift		Rv1668c	
2094911 ACAGCGTCAGCGTCAG	GTTT	Frameshift		Rv1754c	
2109523 CGG	ACAGCGTCAG	Codon deletion	actgagct/act	gnd1	TDA88T
2133468 TTGCGAT	CGGG	Intergenic			
2137521 AC	TTGCGATCCGTCACCTCGAT	Nonsense	gta/gATGCGAGGTGACGGCta	Rv1883c	V73DAR*RL
2207591 T	ACTCCGATCACC	Frameshift		Rv1888c	
2268725 A	TC	Intergenic			
2300205 A	AG	Frameshift		Rv2024c	
	AC	Frameshift		pkx12	

Table S7.3 (cont)

2357268 TGCCGCCGCCGCCGCCG	TGCCGCCGCCGCCGCCG	Frameshift		PE_PGRS36	
2365413 AT	ATT	Intergenic			
2368564 TAA	TA	Intergenic			
2382085 AGTG	AG	Frameshift		PPE37	
2401883 C	T	Intergenic			
2470886 AAACCTAGCCGCGA	AA	Codon change and Codon deletion	aaacttagccgcgag/aag	Rv2206	KLSRE89K
2523205 GCG	GCGCCG	Intergenic			
2525722 CGGGG	CGGGG	Frameshift		Rv2250A	
2617632 A	AT	Intergenic			
2704884 AC	ACAGCGACCATATCGCCGAGCTC	Codon insertion	-/AGCGACCATATCGCCGAGCTC	Rv2407	-64SDHIAEL
2881597 AGGGGGG	AGGGGG	Frameshift		Rv2561	
3028101 ACC	ACCC	Frameshift		Rv2715	
3073868 T	C	Missense	Acc/Gcc	thyA	T202A
3131469 TTGT	TTGTCCGGCGATGT	Codon insertion	-/ACATCGCCG	Rv2823c	-101TSP
3179242 ACGGCGGCGCG	ACGGCGGCG	Codon deletion	gacgcc/gac	Rv2867c	DA20D
3190145 TCCCCC	TCCCC	Frameshift		Rv2880c	
3247856 G	C	Synonymous	cgG/cgC	ppsA	R804
3302324 AGCGAG	A	Frameshift		fadD29	
3415180 ACACCTAGGGGTGGCACCTAGGGGTGGCAC	ACACCTAGGGGTGGCAC	Intergenic			
3473996 GA	GAA	Intergenic			
3480375 GAAA	GAA	Frameshift		Rv3113	
3519294 GGCC	GGCCGCC	Codon change and Codon insertion	cac/cGCCac	nuoH	H6RH
3554066 C	CG	Intergenic			
3580636 CTTT	CTT	Frameshift		lipV	
3590686 GT	GCT	Intergenic			
3595488 CAGCGTAGT	CAGCGTAGTCTGGGAGCGTAGT	Intergenic			
3730741 G	A	Synonymous	ggC/ggT	PPE54	G2065
3884906 A	G	Missense	Aaa/Gaa	Rv3467	K315E
3998059 AGGCGCGCGCGC	AGGCGCGCGC	Codon change and Codon deletion	gcggcc/gcc	PPE64	AA30A
4093879 TGGGGG	TGGGG	Frameshift		PE_PGRS60	
4095001 CG	C	Frameshift		Rv3655c	
4197138 CTT	CTTT	Intergenic			
4198611 CGGGG	CGGGG	Intergenic			
4336090 A	AT	Intergenic			
4338595 GC	G	Intergenic			
4353201 CCCGCCGCC	CCGCCGCC	Codon change and Codon deletion	ccgcc/ca	Rv3876	PP67P
4383144 CC	CCGGGGC	Frameshift		Rv3897c	
4400660 ACCC	ACC	Frameshift		sigM	

<sup>a</sup> Mutated nucleotide is indicated by capitalized letter.

**Table S7.4** - Mutations found to be acquired along node-delimited branches in the Q1 subtree. Position, Reference Sequence and Mutated Sequence are derived from the VCF format.

Branch	Position	Reference Sequence	Mutated Sequence	Type	Codon Change <sup>a</sup>	Affected ORF	Mutation
A-B	49439 C	T	Synonymous	gcG/gcA	Rv0045c	A167	
	176432 A	G	Missense	Acc/Gcc	Rv0149	T245A	
	402528 C	T	Missense	tGc/tAc	aspC	C212Y	
	501882 C	G	Synonymous	gtC/gtG	thiO	V245	
	661798 A	G	Synonymous	ttA/ttG	nrdZ	L168	
	912267 C	G	Missense	Ccg/Gcg	Rv0819	P178A	
	1187439 T	C	Missense	gTt/gCt	Rv1065	V2A	
	1267195 G	A	Intergenic				
	1267253 G	A	Intergenic				
	1295129 C	T	Missense	aCc/aTc	typA	T321I	
	1673425 C	T	Intergenic				
	1771865 C	T	Synonymous	cgG/cgA	Rv1565c	R655	
	1790063 C	T	Intergenic				
	1877638 A	G	Missense	Act/Gct	pkS7	T779A	
	1973157 T	G	Synonymous	ccT/ccG	pknF	P340	
	2058302 A	G	Missense	cAg/cGg	Rv1816	Q16R	
	2088604 G	A	Synonymous	gaC/gaT	PE_PGRS34	D305	
	2204092 G	A	Missense	aCc/aTc	Rv1960c	T45I	
	2211971 G	C	Missense	Gac/Cac	mce3C	D116H	
	2300639 G	A	Synonymous	acC/acT	pkS12	T2116	
	2439519 G	A	Synonymous	cgC/cgT	Rv2177c	R143	
	2584866 G	C	Intergenic				
	2684464 G	A	Intergenic				
	2701886 T	G	Missense	Acc/Ccc	lepA	T455P	
	2780470 A	C	Missense	cTg/cGg	gdh	L598R	
	2964540 G	A	Missense	Ggc/Agc	Rv2638	G46S	
	2974933 A	G	Missense	aTc/aCc	Rv2650c	I101T	
	3401605 G	A	Missense	gCg/gTg	Rv3041c	A105V	
	3593546 T	C	Synonymous	aaT/aaC	Rv3216	N9	
	3694960 C	T	Missense	Cgc/Tgc	pmmB	R33C	
	3792581 G	C	Missense	aCg/aGg	Rv3378c	T223R	
	3992801 C	G	Synonymous	gtC/gtG	fdxB	V39	
	4097203 G	C	Missense	aaC/aaG	Rv3659c	N264K	
	4266215 C	T	Synonymous	ctG/ctA	fbpA	L148	
	4277856 C	T	Missense	cCg/cTg	PE_PGRS62	P429L	
	4365983 T	C	Missense	Acc/Gcc	Rv3884c	T286A	
	B-C	15505 G	C	Missense	Gac/Cac	trpG	D198H
		105060 G	A	Synonymous	gaC/gaT	Rv0095c	D52
		164643 T	C	Synonymous	gtT/gtC	cyp138	V426
		204604 C	T	Synonymous	atC/atT	lprK	I180
236250 G		A	Synonymous	agC/agT	Rv0198c	S86	
490655 T		C	Synonymous	agA/agG	Rv0406c	R17	
507624 T		C	Synonymous	cgA/cgG	Rv0421c	R46	
666566 A		C	Missense	aTg/aGg	Rv0573c	M226R	
1156979 C		A	Missense	cGg/cTg	trcS	R326L	
1274156 C		T	Synonymous	Ctg/Ttg	mmpL13b	L268	
1324915 T		G	Missense	Aat/Cat	Rv1184c	N233H	
1465786 C		T	Synonymous	ctC/ctT	atpG	L301	
1598720 C		A	Missense	gCg/gAg	whiA	A272E	
1674782 T		C	Missense	aTc/aCc	inhA	I194T	
1777422 C		T	Synonymous	Ctg/Ttg	bioF1	L241	
1862460 T		C	Missense	Ata/Gta	PE_PGRS30	I975V	
1888061 A		G	Synonymous	gcA/gcG	pkS9	A12	
1923027 C		T	Missense	Cac/Tac	Rv1698	H95Y	
2009774 CGGG		CGGGG	Frameshift		Rv1775		
2009908 A		G	Missense	aAt/aGt	Rv1775	N246S	
2041559 GC		G	Intergenic				
2193376 C		G	Missense	gaC/gaG	ribA1	D257E	
2443423 C		T	Missense	aCg/aTg	Rv2181	T41M	
2854654 G		A	Synonymous	atC/atT	Rv2530c	I11	
3007095 A		G	Intergenic				
3007148 A		G	Intergenic				
3028101 ACC		ACCC	Frameshift		Rv2715		
3138416 G		A	Missense	Ccg/Tcg	ugpC	P256S	
3141353 C		G	Synonymous	acG/acC	ugpA	T290	
3520172 G		A	Synonymous	ctG/ctA	nuoH	L297	
3652033 TG		TGG	Frameshift		ctpC		
3680075 C		T	Missense	Ccg/Tcg	lhr	P1101S	
4031470 G		T	Synonymous	gtC/gtA	PE_PGRS58	V563	
4182269 C		G	Missense	tCc/tGc	ligC	S171C	
4186821 T		C	Missense	aTg/aCg	Rv3736	M63T	
4195009 G		A	Missense	gCa/gTa	ctpl	A122V	
4198319 A		G	Synonymous	ctT/ctC	Rv3750c	L93	
4216505 C		A	Missense	Gcg/Tcg	Rv3771c	A76S	
4225830 G		A	Synonymous	cgG/cgA	Rv3779	R282	
4247781 T		C	Missense	aTg/aCg	embB	M423T	
4328127 G	C	Synonymous	tcG/tcC	ethR	S193		
4402703 A	C	Missense	AgT/Cgt	trxB2	S326R		
C-D	484379 G	T	Missense	Gcg/Tcg	fadD30	A135S	
	761155 C	T	Missense	tCg/tTg	rpoB	S450L	
	2288868 A	C	Missense	gTc/gGc	pncA	V125G	
	3550815 A	C	Missense	gAt/gCt	Rv3183	D34A	
	3862472 GAA	GA	Intergenic				
D-E	4247429 A	G	Missense	Atg/Gtg	embB	M306V	
D-E	761998 T	C	Missense	cTg/cCg	rpoB	L731P	
E-F	1473246 A	G	rRNA mutation	-	rrs		
F-G	836538 A	G	Missense	Aac/Gac	PE_PGRS9	N280D	
	AGCCGGGTTGGCCGG	AGCCGGGTTGGCCGGTGGCCG	Codon insertion	-/CCGGCCAAC	PE_PGRS15	-602PAN	
	968426 G	G	Codon insertion				
	2365413 AT	ATT	Intergenic				

<sup>a</sup> Mutated nucleotide is indicated by capitalized letter.



**Table S7.5** - Types and number of large SVs ( $\geq 100$  bp) found among the 75 analyzed isolates using the SVMerge pipeline and local assembly validation.

SV Type	No. of variants	Size range (bp)
Deletion	552	100-26351
Deletion + Insertion	8	540-1714
Insertion (incompletely reconstructed)	42	138-1822
Insertion (completely reconstructed)	109	106-562
Inversion	32	258-10493
Complex Inversion	12	1308-8088
Inversion + Deletion	19	1224-9617
Inversion + Insertion	15	212-7114

**Table S7.6** - Multiple comparison test results upon comparison of mean overall  $N_s/S$  and  $T_v/T_s$  ratios for four groups of strains: Lisboa3, Q1, Beijing clades and, other non clustered strains (NC). Significant differences at the 0.05 level are highlighted in bold.

Clades		$N_s/S$ Ratio		$T_v/T_s$ Ratio	
A	B	Mean Difference (A-B)	<i>p</i>	Mean Difference (A-B)	<i>p</i>
Lisboa3	Q1	0.0249	0.4112	-0.0455	<b>0.0330</b>
	Beijing	-0.1156	<b>0.0146</b>	-0.0642	0.1066
	NC	-0.0792	<b>0.0026</b>	-0.0105	0.9773
Q1	Lisboa3	-0.0249	0.4112	0.0455	<b>0.0330</b>
	Beijing	-0.1405	<b>0.0038</b>	-0.0188	0.9673
	NC	-0.1042	<b>0.0001</b>	0.0350	0.2256
Beijing	Lisboa3	0.1156	<b>0.0146</b>	0.0642	0.1066
	Q1	0.1405	<b>0.0038</b>	0.0188	0.9673
	NC	0.0364	0.8487	0.0537	0.2410
NC	Lisboa3	0.0792	<b>0.0026</b>	0.0105	0.9773
	Q1	0.1042	<b>0.0001</b>	-0.0350	0.2256
	Beijing	-0.0364	0.8487	-0.0537	0.2410

**Table S7.7** - Multiple comparison test results upon comparison of mean  $N_s/S$  and  $T_v/T_s$  ratios across the four genomic quadrants. Significant differences at the 0.05 level are highlighted in bold.

Quadrant		$N_s/S$ Ratio		$T_v/T_s$ Ratio	
A	B	Mean Difference (A-B)	<i>p</i>	Mean Difference (A-B)	<i>p</i>
1	2	.3933	<b>.0000</b>	-.0982	<b>.0000</b>
	3	.1041	<b>.0001</b>	.0366	.0987
	4	.0856	<b>.0083</b>	-.0044	.9995
2	1	-.3933	<b>.0000</b>	.0982	<b>.0000</b>
	3	-.2892	<b>.0000</b>	.1348	<b>.0000</b>
	4	-.3078	<b>.0000</b>	.0938	<b>.0000</b>
3	1	-.1041	<b>.0001</b>	-.0366	.0987
	2	.2892	<b>.0000</b>	-.1348	<b>.0000</b>
	4	-.0185	.9871	-.0411	.0614
4	1	-.0856	<b>.0083</b>	.0044	.9995
	2	.3078	<b>.0000</b>	-.0938	<b>.0000</b>
	3	.0185	.9871	.0411	.0614

**Table S7.8** - Multiple comparison test results upon comparison of mean Ns/S and Tv/Ts ratios across the four genomic quadrants for four groups of strains: Lisboa3, Q1, Beijing clades and, other non clustered strains (NC). Significant differences at the 0.05 level are highlighted in bold.

Clades		Quadrant											
		Quadrant 1				Quadrant 2				Quadrant 3			
		Ns/S Ratio		Tv/Ts Ratio		Ns/S Ratio		Tv/Ts Ratio		Ns/S Ratio		Tv/Ts Ratio	
A	B	Mean	Difference (A-B)	p	Mean	Mean	Difference (A-B)	p	Mean	Mean	Difference (A-B)	p	Mean
Lisboa3	Q1	-0.0850	0.3236	0.3818	-0.0497	0.5228	-0.1302	<b>0.0000</b>	0.0409	1.0000	0.8037	<b>0.0000</b>	-0.1787
	Beijing	0.0673	1.0000	0.5228	-0.0509	0.5228	-0.3825	<b>0.0000</b>	-0.0726	0.2574	0.2409	<b>0.0001</b>	-0.1804
	NC	-0.0572	0.6335	1.0000	-0.0158	1.0000	-0.2337	<b>0.0000</b>	0.0184	1.0000	1.0000	<b>0.0180</b>	-0.0872
Q1	Lisboa3	0.0850	0.3236	0.3818	0.0497	0.3818	0.1302	<b>0.0000</b>	-0.0409	1.0000	0.8037	<b>0.0000</b>	0.1787
	Beijing	0.1523	<b>0.0183</b>	1.0000	-0.0012	1.0000	-0.2522	<b>0.0000</b>	-0.1135	<b>0.0150</b>	0.1014	<b>0.0000</b>	-0.0017
	NC	0.0278	1.0000	0.8065	0.0339	0.8065	-0.1035	<b>0.0068</b>	-0.0225	1.0000	0.9465	<b>0.0186</b>	0.0915
Beijing	Lisboa3	-0.0673	1.0000	0.5228	0.0509	0.5228	0.3825	<b>0.0000</b>	0.0726	0.2574	0.2409	<b>0.0001</b>	0.1804
	Q1	-0.1523	<b>0.0183</b>	1.0000	0.0012	1.0000	0.2522	<b>0.0000</b>	0.1135	<b>0.0150</b>	0.1014	<b>0.0000</b>	0.0017
	NC	-0.1245	<b>0.0271</b>	1.0000	0.0351	1.0000	0.1488	<b>0.0038</b>	0.0910	<b>0.0266</b>	0.2940	<b>0.0180</b>	0.0932
NC	Lisboa3	0.0572	0.6335	1.0000	0.0158	1.0000	0.2337	<b>0.0000</b>	-0.0184	1.0000	1.0000	<b>0.0180</b>	0.0872
	Q1	-0.0278	1.0000	0.8065	-0.0339	0.8065	0.1035	<b>0.0068</b>	0.0225	1.0000	0.9465	<b>0.0186</b>	-0.0915
	Beijing	0.1245	<b>0.0271</b>	1.0000	-0.0351	1.0000	-0.1488	<b>0.0038</b>	-0.0910	<b>0.0266</b>	0.2940	<b>0.0186</b>	-0.0932

**Table S7.9** - Multiple comparison test results upon comparison of mean  $N_j/S$  ratios across the different COGs for four groups of strains: Lisboa3, Q1, Beijing clades and, other non clustered strains (NC). Significant differences at the 0.05 level are highlighted in bold.

Clades		COG <sup>a</sup>																				
A	B	C			D			E			F			G			H			I		
		Mean Difference (A-B)	p		Mean Difference (A-B)	p		Mean Difference (A-B)	p		Mean Difference (A-B)	p		Mean Difference (A-B)	p		Mean Difference (A-B)	p		Mean Difference (A-B)	p	
Lisboa3	Q1	0.1074	0.0000		0.2821	0.0292		-0.0869	0.0029		0.0631	0.0448		0.0635	0.9491		-0.1717	0.5064		-0.1300	0.0088	
	NC	-0.1415	0.0026		0.8678	0.0000		-0.2935	0.0001		-0.1314	0.0018		-0.6906	0.2013		-0.2673	0.2588		-0.2348	0.0012	
	Beijing	-0.2203	0.0002		0.7458	0.0088		-0.1664	0.2128		-0.4434	0.0000		-0.7993	0.0338		0.4273	0.0008		-0.3617	0.0000	
Q1	Lisboa3	-0.1074	0.0000		-0.2821	0.0292		0.0869	0.0029		-0.0631	0.0448		-0.0635	0.9491		0.1717	0.5064		0.1300	0.0088	
	NC	-0.2489	0.0000		0.5858	0.0001		-0.2066	0.0081		-0.1944	0.0000		-0.7540	0.1505		-0.0956	0.9844		-0.1048	0.2097	
	Beijing	-0.3278	0.0000		0.4638	0.1068		-0.0796	0.8063		-0.5065	0.0000		-0.8628	0.0213		0.5990	0.0001		-0.2317	0.0025	
NC	Lisboa3	0.1415	0.0026		-0.8678	0.0000		0.2935	0.0001		0.1314	0.0018		0.6906	0.2013		0.2673	0.2588		0.2348	0.0012	
	Q1	0.2489	0.0000		-0.5858	0.0001		0.2066	0.0081		0.1944	0.0000		0.7540	0.1505		0.0956	0.9844		0.1048	0.2097	
	Beijing	-0.0789	0.4273		-0.1220	0.9762		0.1270	0.6569		-0.3120	0.0001		-0.1088	0.9999		0.6946	0.0000		-0.1268	0.2564	
Beijing	Lisboa3	0.2203	0.0002		-0.7458	0.0088		0.1664	0.2128		0.4434	0.0000		0.7993	0.0338		-0.4273	0.0008		0.3617	0.0000	
	Q1	0.3278	0.0000		-0.4638	0.1068		0.0796	0.8063		0.5065	0.0000		0.8628	0.0213		-0.5990	0.0001		0.2317	0.0025	
	NC	0.0789	0.4273		0.1220	0.9762		-0.1270	0.6569		0.3120	0.0001		0.1088	0.9999		-0.6946	0.0000		0.1268	0.2564	
<sup>a</sup> no mutations were found in COG A genes.																						
Clades		COG <sup>a</sup>																				
A	B	J			K			L			M			N			O			P		
		Mean Difference (A-B)	p		Mean Difference (A-B)	p		Mean Difference (A-B)	p		Mean Difference (A-B)	p		Mean Difference (A-B)	p		Mean Difference (A-B)	p		Mean Difference (A-B)	p	
Lisboa3	Q1	0.1190	0.0659		0.0970	0.0255		-0.5724	0.6045		-0.0301	1.0000		0.0834	0.2218		-0.3274	0.0000		0.4746	0.0000	
	NC	0.0048	1.0000		-0.1260	0.5482		-0.6868	0.0910		0.3619	0.0342		-0.3275	0.0000		-0.3546	0.0000		0.5538	0.0000	
	Beijing	0.0468	0.9647		-0.4959	0.0039		0.2742	1.0000		1.0768	0.0000		-0.7615	0.0001		-1.1524	0.0001		1.0010	0.0000	
Q1	Lisboa3	-0.1190	0.0659		-0.0970	0.0255		0.5724	0.6045		0.0301	1.0000		-0.0834	0.2218		0.3274	0.0000		-0.4746	0.0000	
	NC	-0.1142	0.4149		-0.2230	0.0479		-0.1144	1.0000		0.3919	0.1776		-0.4109	0.0000		-0.0272	0.9980		0.0791	0.9374	
	Beijing	-0.0721	0.7210		-0.5929	0.0013		0.8466	0.2502		1.1068	0.0000		-0.8449	0.0000		-0.8250	0.0005		0.5264	0.0000	
Lisboa3	Q1	-0.0048	1.0000		0.1260	0.5482		0.6868	0.0910		-0.3619	0.0342		0.3275	0.0000		0.3546	0.0000		-0.5538	0.0000	
	NC	0.1142	0.4149		0.2230	0.0479		0.1144	1.0000		-0.3919	0.1776		0.4109	0.0000		0.0272	0.9980		-0.0791	0.9374	
	Beijing	0.0420	0.9947		-0.3699	0.0331		0.9610	0.0484		0.7149	0.0000		-0.4339	0.0014		-0.7978	0.0002		0.4473	0.0004	
Beijing	Lisboa3	-0.0468	0.9647		0.4959	0.0039		-0.2742	1.0000		-1.0768	0.0000		0.7615	0.0001		1.1524	0.0001		-1.0010	0.0000	
	Q1	0.0721	0.7210		0.5929	0.0013		-0.8466	0.2502		-1.1068	0.0000		0.8449	0.0000		0.8250	0.0005		-0.5264	0.0000	
	NC	-0.0420	0.9947		0.3699	0.0331		-0.9610	0.0484		-0.7149	0.0000		0.4339	0.0014		0.7978	0.0002		-0.4473	0.0004	

Table S7.9 (cont)

Clades		COG <sup>a</sup>													
A	B	Q		R		S		T		U		V		Not in COG	
		Mean Difference (A-B)	p	Mean Difference (A-B)	p	Mean Difference (A-B)	p	Mean Difference (A-B)	p	Mean Difference (A-B)	p	Mean Difference (A-B)	p	Mean Difference (A-B)	p
Lisboa3	Q1	0.0562	0.4277	0.5745	<b>0.0000</b>	0.0544	0.8148	-0.2211	1.0000	0.0000	-	-2.7872	<b>0.0000</b>	-0.0508	0.3852
	NC	-0.0178	0.9965	0.1572	0.1308	-0.1377	<b>0.0306</b>	-0.4549	1.0000	0.1789	0.9296	-0.4342	0.3460	-0.0784	0.0740
	Beijing	-0.2713	<b>0.0001</b>	0.3293	<b>0.0001</b>	-0.1053	0.7991	-0.9288	0.3723	-0.5714	0.3363	-0.4875	0.3604	-0.0139	1.0000
Q1	Lisboa3	-0.0562	0.4277	-0.5745	<b>0.0000</b>	-0.0544	0.8148	0.2211	1.0000	0.0000	-	2.7872	<b>0.0000</b>	0.0508	0.3852
	NC	-0.0740	0.2977	-0.4174	<b>0.0000</b>	-0.1921	<b>0.0100</b>	-0.2338	1.0000	0.1789	0.9296	2.3529	<b>0.0000</b>	-0.0276	0.9632
	Beijing	-0.3275	<b>0.0000</b>	-0.2452	<b>0.0018</b>	-0.1597	0.4925	-0.7077	0.9821	-0.5714	0.3363	2.2997	<b>0.0000</b>	0.0369	0.9928
NC	Lisboa3	0.0178	0.9965	-0.1572	0.1308	0.1377	<b>0.0306</b>	0.4549	1.0000	-0.1789	0.9296	0.4342	0.3460	0.0784	0.0740
	Q1	0.0740	0.2977	0.4174	<b>0.0000</b>	0.1921	<b>0.0100</b>	0.2338	1.0000	-0.1789	0.9296	-2.3529	<b>0.0000</b>	0.0276	0.9632
	Beijing	-0.2535	<b>0.0001</b>	0.1722	0.1000	0.0324	0.9994	-0.4739	1.0000	-0.7504	0.2214	-0.0533	1.0000	0.0644	0.9130
Beijing	Lisboa3	0.2713	<b>0.0001</b>	-0.3293	<b>0.0001</b>	0.1053	0.7991	0.9288	0.3723	0.5714	0.3363	0.4875	0.3604	0.0139	1.0000
	Q1	0.3275	<b>0.0000</b>	0.2452	<b>0.0018</b>	0.1597	0.4925	0.7077	0.9821	0.5714	0.3363	-2.2997	<b>0.0000</b>	-0.0369	0.9928
	NC	0.2535	<b>0.0001</b>	-0.1722	0.1000	-0.0324	0.9994	0.4739	1.0000	0.7504	0.2214	0.0533	1.0000	-0.0644	0.9130

**Table S7.10** - Multiple comparison test results upon comparison of mean Tv/Ts ratios across the different COGs for four groups of strains: Lisboa3, Q1, Beijing clades and, other non clustered strains (NC). Significant differences at the 0.05 level are highlighted in bold.

Clades		COG <sup>a</sup>											
		C			D			E			F		
A	B	Mean	Difference	p	Mean	Difference	p	Mean	Difference	p	Mean	Difference	p
		(A-B)	(A-B)		(A-B)	(A-B)		(A-B)	(A-B)		(A-B)	(A-B)	
Lisboa3	Q1	0.0073	1.0000		0.1718	0.4818		-0.0890	<b>0.0061</b>		0.0168	0.9100	
	NC	-0.0426	0.3491		-0.0107	1.0000		-0.0555	0.2272		-0.1100	<b>0.0097</b>	
	Beijing	-0.2082	<b>0.0005</b>		0.3312	<b>0.0410</b>		-0.1057	0.2348		-0.1346	<b>0.0089</b>	
	Lisboa3	-0.0073	1.0000		-0.1718	0.4818		0.0890	<b>0.0061</b>		-0.0168	0.9100	
Q1	NC	-0.0498	0.7286		-0.1825	0.4990		0.0335	0.8444		-0.1269	<b>0.0015</b>	
	Beijing	-0.2155	<b>0.0009</b>		0.1594	0.7889		-0.0166	0.9994		-0.1515	<b>0.0043</b>	
	Lisboa3	0.0426	0.3491		0.0107	1.0000		0.0555	0.2272		0.1100	<b>0.0097</b>	
	Q1	0.0498	0.7286		0.1825	0.4990		-0.0335	0.8444		0.1269	<b>0.0015</b>	
Beijing	NC	-0.1656	<b>0.0025</b>		0.3419	<b>0.0385</b>		-0.0501	0.8882		-0.0246	0.9907	
	Lisboa3	0.2082	<b>0.0005</b>		-0.3312	<b>0.0410</b>		0.1057	0.2348		0.1346	<b>0.0089</b>	
	Q1	0.2155	<b>0.0009</b>		-0.1594	0.7889		0.0166	0.9994		0.1515	<b>0.0043</b>	
	NC	0.1656	<b>0.0025</b>		-0.3419	<b>0.0385</b>		0.0501	0.8882		0.0246	0.9907	
<sup>a</sup> no mutations were found in COG A genes; not possible to perform for COG U since some categories had fewer than two cases.													
Clades		J			K			L			M		
		Mean	Difference	p	Mean	Difference	p	Mean	Difference	p	Mean	Difference	p
		(A-B)	(A-B)		(A-B)	(A-B)		(A-B)	(A-B)		(A-B)	(A-B)	
Lisboa3	Q1	-0.4011	<b>0.0410</b>		-0.1486	0.4345		-1.1562	<b>0.0001</b>		-0.2954	<b>0.0000</b>	
	NC	0.0256	0.9999		-0.0617	1.0000		-0.1015	0.9348		0.0395	0.6106	
	Beijing	0.1553	0.4084		-0.1953	0.2497		0.3457	<b>0.0014</b>		-0.0006	1.0000	
	Lisboa3	0.4011	<b>0.0410</b>		0.1486	0.4345		1.1562	<b>0.0001</b>		0.2954	<b>0.0000</b>	
Q1	NC	0.4267	<b>0.0113</b>		0.0869	1.0000		1.0546	<b>0.0001</b>		0.3349	<b>0.0000</b>	
	Beijing	0.5565	<b>0.0012</b>		-0.0467	1.0000		1.5018	<b>0.0000</b>		0.2948	<b>0.0000</b>	
	Lisboa3	-0.0256	0.9999		0.0617	1.0000		0.1015	0.9348		-0.0395	0.6106	
	NC	-0.4267	<b>0.0113</b>		-0.0869	1.0000		-1.0546	<b>0.0001</b>		-0.3349	<b>0.0000</b>	
Beijing	Q1	0.1297	0.0754		-0.1337	0.6823		0.4472	<b>0.0002</b>		-0.0401	0.8351	
	NC	-0.1553	0.4084		0.1953	0.2497		-0.3457	<b>0.0014</b>		0.0006	1.0000	
	Lisboa3	-0.5565	<b>0.0012</b>		0.0467	1.0000		-1.5018	<b>0.0000</b>		-0.2948	<b>0.0000</b>	
	Q1	-0.1297	0.0754		0.1337	0.6823		-0.4472	<b>0.0002</b>		0.0401	0.8351	
Clades		O			N			P			Q		
		Mean	Difference	p	Mean	Difference	p	Mean	Difference	p	Mean	Difference	p
		(A-B)	(A-B)		(A-B)	(A-B)		(A-B)	(A-B)		(A-B)	(A-B)	
Lisboa3	Q1	-0.0250	0.9864		0.0264	1.0000		-0.0250	0.9864		-0.1568	<b>0.0000</b>	
	NC	0.0748	0.2099		0.1588	<b>0.0137</b>		0.0748	0.2099		-0.2175	<b>0.0001</b>	
	Beijing	0.0846	0.1638		0.4371	<b>0.0000</b>		0.0846	0.1638		-0.0086	1.0000	
	Lisboa3	0.0250	0.9864		-0.0264	1.0000		0.0250	0.9864		0.1568	<b>0.0000</b>	
Q1	NC	0.0998	0.1388		0.1324	0.0875		0.0998	0.1388		-0.0606	0.6949	
	Beijing	0.1096	0.1059		0.4107	<b>0.0000</b>		0.1096	0.1059		0.1482	<b>0.0219</b>	
	Lisboa3	-0.0748	0.2099		-0.1588	<b>0.0137</b>		-0.0748	0.2099		0.2175	<b>0.0001</b>	
	NC	-0.0998	0.1388		-0.1324	0.0875		-0.0998	0.1388		0.0606	0.6949	
Beijing	Q1	0.0098	0.9999		0.2783	<b>0.0003</b>		0.0098	0.9999		0.2088	<b>0.0046</b>	
	NC	-0.0846	0.1638		-0.4371	<b>0.0000</b>		-0.0846	0.1638		0.0086	1.0000	
	Lisboa3	-0.1096	0.1059		-0.4107	<b>0.0000</b>		-0.1096	0.1059		-0.1482	<b>0.0219</b>	
	Q1	-0.0098	0.9999		-0.2783	<b>0.0003</b>		-0.0098	0.9999		-0.2088	<b>0.0046</b>	

Table 7.10 (cont)

Clades		Q			R			S			T			V			Not in COG		
A	B	Mean Difference (A-B)	p		Mean Difference (A-B)	p		Mean Difference (A-B)	p		Mean Difference (A-B)	p		Mean Difference (A-B)	p		Mean Difference (A-B)	p	
Lisboa3	Q1	-0.0904	<b>0.0017</b>		-0.0328	0.5666		-0.1114	0.2938		-0.1139	0.1037		-0.3456	<b>0.0001</b>		0.0595	<b>0.0011</b>	
	NC	-0.0589	0.2656		0.0571	<b>0.0339</b>		0.0398	1.0000		0.1728	<b>0.0068</b>		-0.5103	<b>0.0001</b>		-0.0171	0.9612	
	Beijing	-0.0924	0.4650		-0.1261	<b>0.0123</b>		-0.1703	0.0594		0.2943	<b>0.0428</b>		-0.4182	<b>0.0001</b>		-0.1598	0.1011	
Q1	Lisboa3	0.0904	<b>0.0017</b>		0.0328	0.5666		0.1114	0.2938		0.1139	0.1037		0.3456	<b>0.0001</b>		-0.0595	<b>0.0011</b>	
	NC	0.0315	0.8461		0.0899	<b>0.0148</b>		0.1512	<b>0.0117</b>		0.2867	<b>0.0002</b>		-0.1646	0.6417		-0.0766	<b>0.0034</b>	
	Beijing	-0.0020	1.0000		-0.0932	0.0882		-0.0589	1.0000		0.4082	<b>0.0060</b>		-0.0725	0.8580		-0.2194	<b>0.0235</b>	
NC	Lisboa3	0.0589	0.2656		-0.0571	<b>0.0339</b>		-0.0398	1.0000		-0.1728	<b>0.0068</b>		0.5103	<b>0.0001</b>		0.0171	0.9612	
	Q1	-0.0315	0.8461		-0.0899	<b>0.0148</b>		-0.1512	<b>0.0117</b>		-0.2867	<b>0.0002</b>		0.1646	0.6417		0.0766	<b>0.0034</b>	
	Beijing	-0.0335	0.9888		-0.1831	<b>0.0004</b>		-0.2101	<b>0.0026</b>		0.1215	0.7305		0.0921	0.9591		-0.1427	0.1646	
Beijing	Lisboa3	0.0924	0.4650		0.1261	<b>0.0123</b>		0.1703	0.0594		-0.2943	<b>0.0428</b>		0.4182	<b>0.0001</b>		0.1598	0.1011	
	Q1	0.0020	1.0000		0.0932	0.0882		0.0589	1.0000		-0.4082	<b>0.0060</b>		0.0725	0.8580		0.2194	<b>0.0235</b>	
	NC	0.0335	0.9888		0.1831	<b>0.0004</b>		0.2101	<b>0.0026</b>		-0.1215	0.7305		-0.0921	0.9591		0.1427	0.1646	



**Table S7.11** – End sequences from the different ISs used as probes to extract reads for mapping analysis.

IS	5' End	3' End
IS6110	TGAACCGCCCCGGCATGTCCGGAGA	TCTCCGGACTCACCGGGGCGGTTCA
IS1532	GTGGCCACGATAGCCCAACGGCTGC	CAAGAGACCCGGCCGACCAACCAGC
IS1533	AATGTTGACTGTGGAAGATTGGGCT	CCACCAGCCGGAACCACCGAAGAAT
IS1537	GTTTTGAGTGGTCGGTAGTTGTCGG	ACTAAAGATCGCTCGCTTGCAACGG
IS1538	TGAGTGGTCGGTAGTTGTCACTGGCGTGCG	none <sup>a</sup>
IS1539	ATGTCAAGGATACTGACACATGTGC	CCGAGACGGGGTGCAAGTCAAGTGA
IS1540	CGTGATCGACACGGCCATCGAGGAG	CTCCTGCCATCGGGGCAGATACGCT
IS1552	GGTGTTTGCCGAGCTGATCCGTGCC	GTCGCCGACCCTCTCTGTCAACCTT
IS1553	GAGATCGTCGGTGTGTACCGCCAAG	GCGGCCAGTAGGCACCGACGAAGT
IS1556	TGACACATGACCGCTTCCGCCGCC	TTTTGCGGTTGATGGATGGTAGCTG
IS1560	TTGATCCCCGGTCGGATGGTGCTGA	GGTCACGATCAGCGCCCTCCCCGCA
IS1561	GTGGCCATCGACCCCGCCGCTGCCT	none <sup>a</sup>
IS1602	none <sup>a</sup>	AGCACGCCACTGACAACTACCGACCACTCA
IS1603	GTGCGCCAAATTAGTAGTCGCTATC	ACCAGACCATCAATTGTTGCGACGT
IS1604	GGTGGCGGTGCGCGATGACGAGGAG	ACCGGCCAGGAGGCCCAACCGAAAT
IS1605	TTGGGCCCATCGTCGAAAACCTGCC	TAGTCGCGTGACCACTAAAGATCACTCACTTGCAACGG
IS1606	GGTGACCCGCGATCCACACAGCCCG	ATCGAGCCTTAAGGAAAATCAAAGC
IS1607	GCGGCCCAAACCACTACCCTGCCCCG	CCTCCAGGCACTGCTCAACCAAGAT
IS1608	GTGACTGCCGAGAATCCAGGACGGA	GCGTCGGTTGATGTCGTTGGCGCGG
ISB9	ATCACCCCGGCAAGCTGTCCCTGAG	GCCGAGCATCTCTTTCGCGGGTGAT

<sup>a</sup> Sequences at this end did not allow the unambiguous mapping of the respective Insertion Sequence